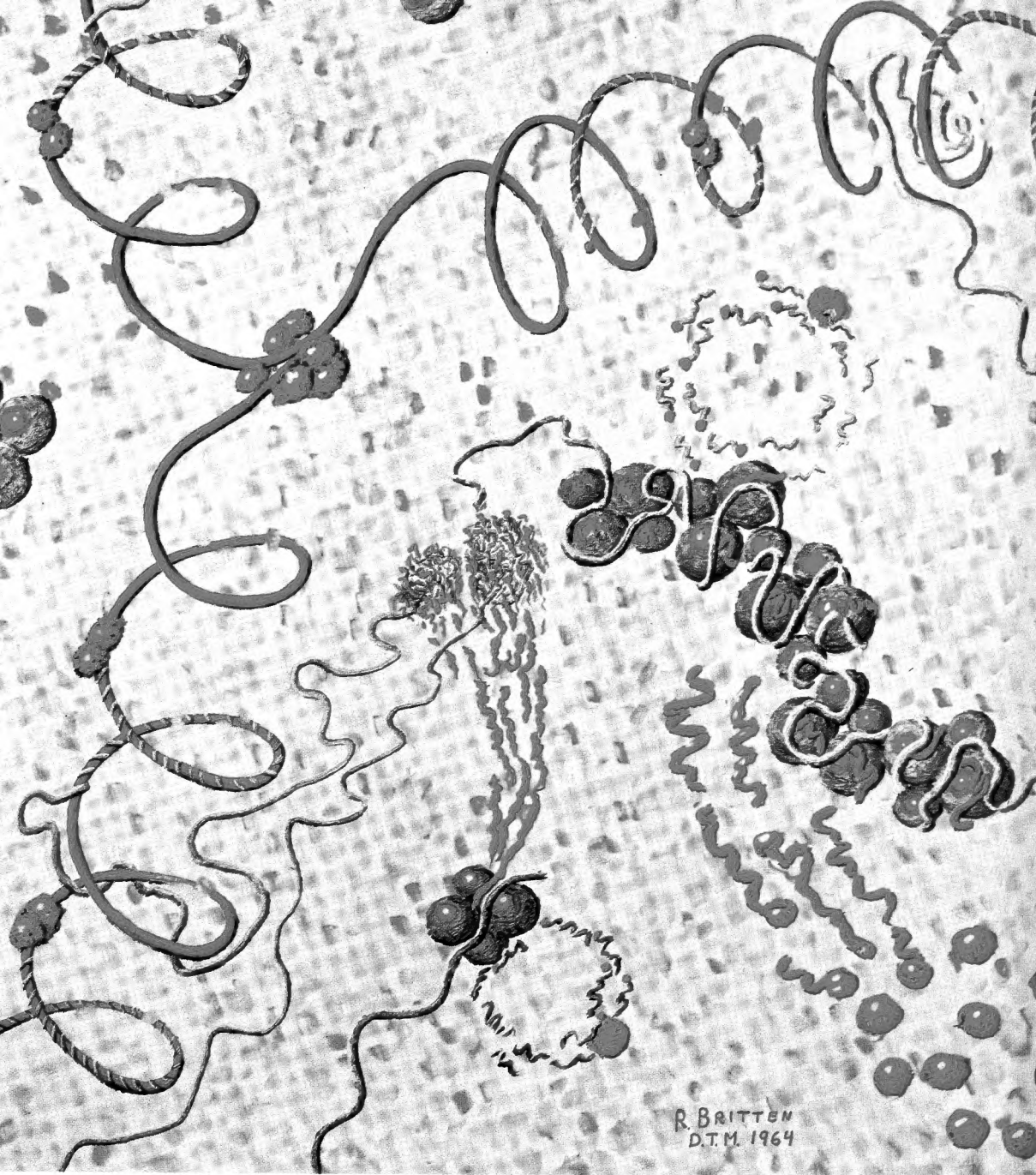


Studies of
MACROMOLECULAR
BIOSYNTHESIS

Edited by
RICHARD B. ROBERTS

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Macromolecular Metabiology

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PREFACE

In these days of massive scientific effort, we hear much of scientific planning, and are deeply concerned about it. In an era when the national expenditure for science and engineering is projected at some fifteen billion dollars, when nearly a twentieth of all working men and women in the United States are technical specialists of one sort or another, and when the federal government is supporting more than two-thirds of all the research and development in the country, it is small wonder that we should be so preoccupied with questions of how we can best utilize our comparatively limited scientific and technological resources. Planning for science, indeed, has become an abiding consideration with us in the last years, even though we do not see very clearly now how it should--or, indeed, can--be done.

There is merit in this preoccupation. But it also involves a very serious and present peril. That danger is that we may confuse this search for ways of planning for science with the far deeper processes of planning in science--the processes through which, in the end, all real scientific growth takes place; the processes which, in the final analysis, alone can bring about true scientific growth. Planning for science involves planning for the known--a process that, in the final analysis, is the opposite of scientific discovery. Planning within science is but the final and retrospective sum of the acts of true understanding that, from time to time and place to place over the scientific front, lead from one conceptual level to another. These are the processes through which the real shaping of science will continue in the future as it has always occurred in the past. The greatness of scientific scholarship, and the molding of its future, inheres only in that thin, tenuous thread of reason and vision and insight and crucial experiment by which new fields have always been conceived and developed. In times of massive scientific effort undertaken at an unprecedented rate, and on an unprecedented scale, it is especially important that this cardinal fact be remembered. Especially vivid and striking proof of it is given in the following pages.

Today we speak of molecular biology as a "field," now that it has brilliant achievements to its credit. We are beginning to plan for it on the national scene. But it is easy to forget that its importance, and indeed its very existence today, depends in large measure on the fact that a rather small number of research workers in the physical and biological sciences, casting about hardly more than three decades ago in search of new areas of inquiry adequately challenging, drew bead on fundamental biology. The pioneers in this effort began their work well before the last war. But at the close of the war their numbers

were importantly augmented, as fresh minds, released from research in defense of the nation, joined their ranks. This, as it turned out, was major scientific planning. But it was planning in its traditional scientific sense--not only planning of highly individual character, but planning conducted from day to day and at the laboratory bench--planning, in short, at the level of the work.

It is of great interest and significance that these pioneering advances took place predominantly among small groups of gifted individuals in environments where freedom and flexibility were at a maximum. It is of much interest, too, that many of the men and women participating in the search were trained in quite different fields and that their earlier deepest experiences commonly lay in one or another of the physical sciences. A number of examples of this situation could be quoted. A particularly classic one is offered by the group in the Laboratory of Molecular Biology of the British Medical Research Council at Cambridge. That group, initiated shortly after the war with two crystallographers, has never numbered more than a handful of workers. Yet within the last five years it has been a major factor in bringing about two of the greatest advances of modern times in our understanding of fundamental biological processes: our comprehension of the basic structure of proteins and of the nature of the genetic code.

In the following pages is set forth the detailed story of the growth and development and some of the accomplishments of another classic group of this kind, having a somewhat similar background and pioneering in similar fields. The Biophysics Group of the Department of Terrestrial Magnetism of the Carnegie Institution of Washington was formally constituted in 1946, just after the close of World War II, and it, too, was composed in considerable measure of investigators whose primary training and work had been in quite different fields. It had longer and deeper roots, however, growing from a program of biophysics initiated in the Department as early as 1932 by the discoverer of radar--later to become Director of the Department itself--and his physician wife. Its first formal Chairman was the codiscoverer of neptunium, who in the war years had pioneered the designs for atomic-powered undersea craft which were later to show such spectacular development. Its second, and present, Chairman is a nuclear physicist by original commitment who during the war was deeply concerned, among other things, with the development of guided missiles. A senior staff member had been an early leader in cyclotron design. Intimate associations were formed with a number of biologists, as guest investigators or in their own universities. But it was three years before a member whose primary training lay in the field of biology at the level of biophysics and biochemistry joined the group permanently, and even he had had extensive training in physics and had worked in the design and the vital use of physical military instruments during the war.

The achievements of that group in advancing our understanding of biological processes at the deepest levels cannot be summed up at present. They are currently in midstream, and some of the most exciting of all may still lie over the horizon. But the conquests detailed in the following pages are already formidable. An early concentration upon studies of the biosynthesis of small molecules in microorganisms provided an intensive focus of common effort which set a lasting stamp upon the direction of thought and work. It also provided a major point of departure: to new and fundamental concepts about the ways in which these small molecules are themselves assembled into the larger ones so vital to all life processes; to the coupling between the synthesis of protein and that of nucleic acids; to the formation of enzymes. From such advances of understanding have come in the very last years spectacular discoveries related to the synthesis and the nature of RNA itself and of the modes of action of messenger RNA, and, most recently, of the relation of virus to host in organisms; and yet more broadly, and perhaps of even wider significance, findings connecting the taxonomic relatedness of different organisms with the relatedness of the genetic coding in the DNA of their chromosomes. So recent are these latest advances, and still in so formative a stage, that their detailing must lie beyond the scope of this monograph. It is hard to predict what the future may bring, except that it is almost certain to be highly novel, deeply exciting, and fundamentally important.

But this monograph describes more than the specific development of an exciting program of investigation. It embodies the very natural history of a group of investigators which, as its own history has amply demonstrated, is peculiarly fitted to conduct work of the highest originality along a new borderline between the sciences. It has much to say about the optimum environment for such work--the optimum size of such a group, its optimum composition, the optimal organizational arrangements surrounding it. It has a great deal to say about the freedom and flexibility of the individual that is so vital a feature of such a research environment.

Yet vitally important as the maintenance of the independence of the individual has been to such an effort, it is significant that the group itself has been far more than their sum. In a very real sense it is an organism in itself--a sensitive, labile organism, building in a highly mobile and flexible and important way upon the joint contributions of its members. Some of the factors contributing to that end are described in this monograph. Among them, for example, have been some modes of approach: the concentration of the whole group upon rather narrow, quite specific, and extremely fundamental problems punctuated by periods of quite the opposite activity, of wide-ranging exploratory efforts to seek new points of concentration; the continuing absence of formal program; the unremitting search for underlying patterns. The size and composition of the group have clearly been of first importance. But one ingredient not men-

tioned in the monograph itself should, I think, be emphasized here because it is so important. In the whole history of this work it has again and again proved impossible to trace the origins of many of the most important ideas or to assign them to any one individual. That has come about in large measure because of an outstanding quality permeating the whole atmosphere of the group and dominating the contributions of its members--the luminous quality of the grace of giving. Caryl P. Haskins.

Washington, D. C.
June 1964

CONTENTS

I.	Foreword	1
II.	The precursor pools	3
A.	Introduction, R. J. Britten, D. B. Cowie, and R. B. Roberts	3
B.	<u>E. coli</u> amino acids	5
	1. Amino acid adsorption and protein synthesis in <u>Escherichia coli</u> , R. J. Britten, R. B. Roberts, and E. F. French	5
	2. The amino acid pool in <u>Escherichia coli</u> , Roy J. Britten and F. T. McClure	13
	3. Protoplasts, E. T. Bolton and J. J. Leahy	57
C.	<u>E. coli</u> nucleotide pools	61
	1. Phosphorus incorporation, E. T. Bolton and R. B. Roberts	61
	2. The synthesis of ribosomes in <u>E. coli</u> , 1, The incorporation of C ¹⁴ -uracil into the metabolic pool and RNA, B. J. McCarthy and R. J. Britten	71
	3. Incorporation of ribonucleic acid bases into the metabolic pool and RNA of <u>E. coli</u> , M. Buchwald and R. J. Britten	84
D.	Yeast pools	96
	1. Kinetics of formation and utilization of metabolic pools in the biosynthesis of protein and nucleic acid, Dean B. Cowie and Barbara P. Walton	96
	2. The use of metabolic pools of purine compounds for nucleic acid synthesis in yeast, Dean B. Cowie and Ellis T. Bolton	112
	3. Metabolic pools and the synthesis of macromolecules, Dean B. Cowie and Frank T. McClure	119

4. Metabolic pools and the utilization of amino acid analogs for protein synthesis, Ellis S. Kempner and Dean B. Cowie	129
E. Summary of <u>E. coli</u> pools	137
F. Summary of yeast pools	142
III. Ribosomes	147
A. General properties of ribosomes	147
1. Excerpt from 'Kinetic studies of the synthesis of RNA and ribosomes,' R. B. Roberts, R. J. Britten, and B. J. McCarthy	147
B. Kinetic studies of the synthesis of RNA and ribosomes	169
1. The synthesis of ribosomes in <u>E. coli</u> , 2, Analysis of the kinetics of tracer incorporation in growing cells, R. J. Britten and B. J. McCarthy	169
2. The synthesis of ribosomes in <u>E. coli</u> , 3, Synthesis of ribosomal RNA, B. J. McCarthy, R. J. Britten, and R. B. Roberts	176
3. The synthesis of ribosomes in <u>E. coli</u> , 4, The synthesis of ribosomal protein and the assembly of ribosomes, R. J. Britten, B. J. McCarthy, and R. B. Roberts	202
4. The nucleotide base composition of ribonucleic acid from several microbial species, J. E. M. Midgley	212
5. The synthesis and kinetic behavior of deoxyribonucleic acid-like ribonucleic acid in bacteria, J. E. M. Midgley and B. J. McCarthy	225
6. A general method for the isolation of RNA complementary to DNA, E. T. Bolton and B. J. McCarthy	247
C. Related papers	255
1. Stability of ribonucleoprotein particles of <u>Escherichia coli</u> , Ellis T. Bolton, Bill H. Hoyer, and Daniel B. Ritter	255

2. Fractionation of <u>Escherichia coli</u> for kinetic studies, Richard B. Roberts, Roy J. Britten, and Ellis T. Bolton	259
3. Ribosome synthesis during unbalanced growth, Fred C. Norcross, Lucy T. Comly, and Richard B. Roberts	270
4. High-resolution density gradient sedimentation analysis, R. J. Britten and R. B. Roberts	273
5. Variations in bacterial ribosomes, B. J. McCarthy	276
6. Sedimentation characteristics of bacterial ribonucleo-protein obtained at different periods during the cell-division cycle, R. J. Britten, K. G. Lark, and F. Norcross	278
7. Structure of ribosomes from <u>Escherichia coli</u> as revealed by their disintegration, M. Beer, P. J. Highton, and B. J. McCarthy	280
8. Studies of <u>E. coli</u> ribosomal RNA and its degradation products, Arthur I. Aronson and Brian J. McCarthy	284
9. The kinetics of the synthesis of ribosomal RNA in <u>E. coli</u> , Brian J. McCarthy and Arthur I. Aronson	299
10. Hydrolysis of RNA by lead acetate, R. Britten	318
11. The effects of magnesium starvation on the ribosome content of <u>Escherichia coli</u> , B. J. McCarthy	328
12. Alternative codes and templates, Richard B. Roberts	337
13. Further implications of the doublet code, Richard B. Roberts	341
14. The kinetics of transfer ribonucleic acid synthesis in <u>Escherichia coli</u> , J. E. M. Midgley	347
D. Related material from Carnegie Year Books	358
1. Sedimentation constants of ribosomes, R. J. Britten and B. J. McCarthy	358
2. Ribonuclease--self-digestion RNase, E. T. Bolton	362
3. Leucine aminopeptidase, E. T. Bolton and B. J. McCarthy	365

4. Composition of the RNA, E. T. Bolton	368
5. Particles of other microorganisms, B. J. McCarthy	370
6. Nucleotide sequences in <u>E. coli</u> ribonucleic acids, E. T. Bolton	371
7. Studies with <u>Tetrahymena</u> , R. J. Britten	376
8. Specific yeast RNA analysis, J. E. M. Midgley	381
9. DEAE chromatography of ribosomes, B. J. McCarthy	384
IV. Protein synthesis	387
A. Correlation with ribosomes	387
1. Synthesis of nascent protein by ribosomes in <u>Escherichia coli</u> , Kenneth McQuillen, Richard B. Roberts, and Roy J. Britten	387
2. Ribosome-bound β -galactosidase, D. B. Cowie, S. Spiegelman, R. B. Roberts, and J. D. Duerksen	398
3. Ribosomal enzymes, J. D. Duerksen and D. B. Cowie	407
4. Kinetic studies of β -galactosidase induction, J. A. Boezi and Dean B. Cowie	408
5. A model for the mechanism of enzyme induction, R. B. Roberts, R. J. Britten, and F. T. McClure	417
B. Analogs	
1. The effects of 6-mercaptopurine on biosynthesis in <u>Escherichia coli</u> , Ellis T. Bolton and H. George Mandel	424
2. Remplacement total de la méthionine par la sélénométhionine dans les protéines d' <u>Escherichia coli</u> , Georges N. Cohen and Dean B. Cowie	436
3. Biosynthesis by <u>Escherichia coli</u> of active altered proteins containing selenium instead of sulfur, Dean B. Cowie and Georges N. Cohen	440

4. Amino acid analog incorporation into bacterial proteins, Dean B. Cowie, Georges N. Cohen, Ellis T. Bolton, and Huguette de Robichon-Szulmajster	450
5. The effect of 5-fluorouracil on bacterial protein and ribonucleic acid synthesis, Arthur I. Aronson	458
6. 5-Fluorouracil, J. E. M. Midgley	468
C. Other aspects of protein synthesis	472
1. The effects of a tryptophan-histidine deficiency in a mutant of <u>Escherichia coli</u> , Margot K. Sands and Rich- ard B. Roberts	472
2. Role of peptides in protein synthesis, R. B. Roberts	478
3. The induced synthesis of β -galactosidase in <u>E. coli</u> , 1, Synthesis of enzyme under various experimental con- ditions, Søren Løvtrup	480
4. The induced synthesis of β -galactosidase in <u>E. coli</u> , 2, Analysis of the accompanying synthetic activity by means of isotopes, Søren Løvtrup	489
5. Osmotic upshock, R. B. Roberts	496
6. The formation of protomorphs, Frank T. McClure and Richard B. Roberts	497
7. Soluble RNA, E. T. Bolton and R. B. Roberts	503
8. Studies with the mutant <u>E. coli</u> 15 T ⁻ A ⁻ U ⁻ , D. B. Cowie, B. J. McCarthy, and R. B. Roberts	506
9. Control mechanisms, effects of virus infection, D. B. Cowie	515
V. Supplementary material	519
A. Virus	519
1. Mammalian viruses and rickettsiae--their purification and recovery by cellulose anion exchange columns, Bill H. Hoyer, Ellis T. Bolton, Richard A. Ormsbee, George LeBouvier, Daniel B. Ritter, and Carl L. Larson	519

2. Simple method for preparation of purified radioactive poliovirus particles: electron micrograph, Bill H. Hoyer, Ellis T. Bolton, Daniel B. Ritter, and Edgar Ribi	527
3. Kinetics of labelling of turnip yellow mosaic virus with P^{32} and S^{35} , R. E. F. Matthews, E. T. Bolton, and H. R. Thompson	530
B. <u>Hydra</u>	541
1. Migration of ^{14}C -labeled cnidoblasts, H. M. Lenhoff	541
2. Digestion of protein in <u>Hydra</u> as studied using radioautography and fractionation by differential solubilities, H. M. Lenhoff	544
C. Mouse	563
1. Biochemical and physiological differentiation during morphogenesis, 22, Observations on amino acid and protein synthesis in the cerebral cortex and liver of the newborn mouse, Louis B. Flexner, Josefa B. Flexner, and Richard B. Roberts	563
2. Biochemical and physiological differentiation during morphogenesis, 23, Further observations relating to the synthesis of amino acids and proteins by the cerebral cortex and liver of the mouse, R. B. Roberts, J. B. Flexner, and L. B. Flexner	582
3. Lactic dehydrogenases of the developing cerebral cortex and liver of the mouse and guinea pig, L. B. Flexner, J. B. Flexner, R. B. Roberts, and G. De La Haba	594
4. Inhibition of protein synthesis in brain and learning and memory following puromycin, J. B. Flexner, L. B. Flexner, E. Stellar, G. De La Haba, and R. B. Roberts	608
5. Memory in mice as affected by intracerebral puromycin, Josefa B. Flexner, Louis B. Flexner, and Eliot Stellar	619
VI. Ribosomes 1964	624
A. Introduction	624
B. Precursor-product relationships	625

C.	Quantities of cellular materials	627
D.	Rates of nucleic acid synthesis	633
E.	Sites of nucleic acid synthesis	633
F.	Limitations on synthetic rates	636
G.	The role of ribosomes in protein synthesis	638
H.	Rates of protein synthesis	639
I.	Polysomes	640
J.	The synthesis of β -galactosidase	642
K.	The synthesis of ribosomal protein	643
L.	Some current problems	647
M.	References	653
VII.	Brief historical sketch	656
A.	The good old days	656
B.	The beginnings of the Biophysics Section	657
C.	The formulation of a program	659
D.	Permeability	667
E.	Synthesis of small molecules in the intact cell	674
F.	Pools	675
G.	Ribosomes	679
H.	Conclusion	687
I.	References	689
	Appendix 1	691
	Appendix 2	692

I. FOREWORD

Research is not complete until the results are disseminated and integrated into the scientific record. The research scientist must therefore attempt to ensure effective communication of his work. We have utilized a number of techniques in trying to achieve this result. Satisfying answers to significant questions have been published in the usual professional journals. Progress reports describing our work for the year in considerable detail have appeared regularly in the Carnegie Year Books. We have considered the Year Books an especially useful channel of communication because they include in each report all the aspects of our laboratory work and they describe various endeavors that have not yet achieved (and may never achieve) the degree of completeness we consider necessary for contributions to journals. They also provide a record of the train of thought motivating our experimental efforts.

We believe the "grapevine" to be another important mode of communication, and we welcome visitors in the laboratory either for an afternoon or for longer periods. Equally, we find our visits to other laboratories most rewarding. We believe that in these exchanges all aspects of our work, past, present, and future, are suitable for discussion. The danger of seriously misleading any competent observer by showing him unconfirmed results and tentative conclusions seems trivial.

Finally, there are the more organized techniques for direct communication: meetings, symposia, and conferences. We have participated in these affairs and have contributed to the volumes emerging from some of them.

As a result of all these activities, roughly 1000 pages of published material have accumulated in eight years. Nevertheless we do not consider that our responsibility for effective communication of our results has been fulfilled. The material is scattered widely through different publications, and there has been no opportunity to view old results with the benefit of hindsight or even to correct errors.

Faced with a similar situation in 1953 we decided to prepare a book (Studies of Biosynthesis in E. coli) presenting our results as a unified whole. This effort consumed a year's work of the whole laboratory, partly in writing and partly in new experimental work to fill in gaps that became obvious during the writing. The outcome was rewarding both because the experimental data

were made much more readily available to other investigators and because the results were more meaningful when placed in a coherent framework.

At the time that book was started the magnitude of the task ahead was fortunately not apparent. The work involved in preparing a similar volume for our work on ribosomes and protein synthesis is only too obvious, and we are unwilling to divert the required time from our present activities.

The present book is therefore a compromise. We have sacrificed continuity, unity, and cohesion as criteria impossible to meet with the time and effort available. Our principal concern has been to assemble the original publications in a convenient and available form, since most of this material is no longer available as reprints. We have included comments on some of the papers which correct errors or point out significant items that were not appreciated at the time of writing. One new section is added to evaluate current concepts in the areas of molecular biology in which we have been involved. An historical sketch is added partly out of nostalgia and partly to trace the progress of a group of physical scientists in their attempts to explore the reaches of biology.

To reduce the 1000 pages to manageable proportions we have omitted almost all contributions to symposia, reviews, and general articles. Only the parts of the Year Books are included that we think significant and that have not been published elsewhere.

We trust that no reader will even attempt to read from the beginning to the end of this volume. Rather, we hope that the table of contents will direct him to some items that are useful or interesting, or possibly both.

II. THE PRECURSOR POOLS

A. Introduction

Today many features of macromolecular synthesis seem as familiar as old friends. Colorful diagrams illustrating the most intimate details have been published in popular magazines. In 1954, however, the situation seemed almost hopelessly complicated. The magnitude of the problem was well known. An organism as small as a bacterium--roughly 10^{-12} ml in volume--carried all the machinery needed to replicate its complements of DNA, RNA, and protein. Protein synthesis was under the direction of DNA, but RNA seemed to be implicated too. Although the processes were accurate and few mistakes were made, they were also extremely rapid. Radioactive tracers could be found in macromolecules within seconds after they were supplied to growing cultures, and the cell could duplicate itself in 20 minutes.

Conceptually, at least, the process could be visualized as occurring in discrete stages. Molecules enter the cells. They undergo various chemical modifications. They are selected for incorporation into new macromolecules in the proper position. They are polymerized to form the new chains. Subsequently, the newly formed polymers move from the sites of synthesis and undergo further alterations before reaching their final forms.

Information on the process could be sought either by observing the state of the small molecules immediately before incorporation into macromolecules or by observing the properties of the newly polymerized materials. In 1954 the available techniques were far better suited for the characterization of the small molecules, and a start was made on the studies of the precursor pools, which are described in this section. Quite different techniques had to be developed for the fractionization of larger molecules needed for the second type of study; they are described in parts III and IV.

At that time yeast was known to accumulate large reserves of amino acids that could be utilized for protein synthesis. The kinetics of tracer incorporation had already showed delays that could be attributed to the use of endogenous material. Furthermore, the theory that small peptides were linked into larger ones, finally forming proteins, was still widely supported. Some of our first efforts were directed toward determining whether the yeast had peptides in their metabolic pools and whether such peptides were intermediates in protein synthesis or merely reserves.

Escherichia coli, in contrast, was believed to contain few if any free amino acids. Amino acids present might be those adsorbed to RNA acting as a template for protein synthesis. Thus both the template theory and the peptide theory provided good reasons for examining the nature of the precursor pools.

The shortcomings of these concepts soon became apparent. Neither the yeast nor the bacteria contained significant quantities of peptides. E. coli was found to concentrate quantities of amino acids far beyond the adsorptive capacity of the assumed templates. By that time the complexities of the pools and the need for further investigation of their roles in determining the intracellular environment were apparent.

Probably the most significant of the many unexpected features that have turned up is the heterogeneity of the pools. In either yeast or E. coli relatively large quantities of amino acids can be stored by the cells in more than one way. In yeast many of the biosynthetic events seem to be restricted to the molecules of one type of pool.

In addition there must exist a variety of states of more transient nature, such as association with synthetic or regulatory sites. An elaborate organization of the cell for efficient performance of its specialized functions is revealed.

The studies of pools to date indicate the need to postulate these properties; further work on pools is necessary for a more complete understanding. These features of the living cell may be those that endow it with rates of protein synthesis a thousand times higher than the cell-free systems in use today.

The original hope of gaining direct insight into the processes of protein and nucleic acid synthesis by observing the progress of molecules through the precursor stages was partially fulfilled. The formation of small peptides did not appear to be a significant step in protein synthesis. Conversion of amino acids occurred before, not after, insertion into a polypeptide chain. The mechanism of pool formation provided discrimination against unnatural amino acid analogs. The experience gained, moreover, proved essential for the studies of the newly formed polymers. For proper design of the experiments the kinetic properties of the pools must be known; otherwise, delays in passing through the pool may obscure the phenomenon of interest. Similarly, the delays introduced by the pool, the conversions, and other peculiarities must be appreciated, or features introduced by the pool may be attributed to quite different causes.

Roy J. Britten, D. B. Cowie, and Richard B. Roberts.

B. E. coli Amino AcidsII.B.1 Amino Acid Adsorption and Protein Synthesis in Escherichia coli

(Reprinted, by permission, from Proceedings of the National Academy of Sciences, vol. 41, no. 11, pp. 863-870, November 1955.) (Read before the Academy, April 25, 1955; communicated by M. A. Tuve, July 20, 1955.)

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Washington, D. C.

Two quite distinct theories of protein synthesis are currently popular. One holds that amino acids are linked into small peptides which then serve as building blocks for the proteins. The other postulates that amino acids are individually adsorbed on a large template molecule and are then linked together into the polypeptide chains. This paper reports the results of studies of amino acid incorporation by growing *Escherichia coli*. The results furnish experimental evidence in favor of the template theory.

When *E. coli* cells (grown in a medium¹ containing glucose, salts, and ammonia) are washed and treated with cold 5 per cent trichloroacetic acid (TCA), a number of amino acids are extracted. These amino acids are transient intermediates of synthesis. When C¹⁴-glucose is added to the medium, their specific radioactivity rises more rapidly than that of the cell as a whole. They equally rapidly lose their radioactivity when the C¹⁴-glucose is replaced by C¹²-glucose. The radioactivity thus lost by the TCA-soluble fraction of the cells is transferred to the protein fraction. It therefore appeared that a study of the TCA-soluble fraction and its kinetic relationship to the protein fractions of the cell might throw some light on the mechanisms of protein synthesis.

The quantity of individual amino acids occurring in the TCA-soluble fraction depends on the conditions of growth of these cells. In the absence of supplements, glutamic acid, alanine, and valine are major components, while traces of other amino acids are present, and proline and methionine cannot be detected. When amino acids (including proline and methionine) are added as supplements to the medium, they appear in the TCA-soluble fraction.

Since *E. coli* is highly permeable to amino acids, it appears that the amino acids extracted by TCA are held within the cell by a loose binding which can be broken by 5 per cent TCA. For simplicity in discussion these transient loosely bound amino acids will be referred to as "adsorbed" even though neither the site nor the nature of the binding has been identified.

The time course of adsorption and incorporation of amino acids into protein can be measured accurately by adding to growing cultures of bacteria small quantities of highly radioactive amino acids (25 per cent C^{14}). To measure the total uptake of radioactivity, samples of bacteria were harvested by passing the suspension through porous collodion membrane filters, a process requiring only 5–10 seconds. To measure the incorporation into the protein, samples of the cell suspension were injected into an equal volume of 10 per cent TCA and after 15 minutes at room temperature were filtered as described above. The incorporation of radioactivity into the whole cell and into the TCA-precipitable fraction could thus be measured under identical conditions. The difference gives the uptake into the TCA-soluble fraction of the cell and measures the adsorbed quantity of amino acid. Measurements have been made of the quantity of amino acid adsorbed on the filter in the absence of cells, and, where necessary, appropriate corrections have been made.

A typical experiment proceeds as follows: The tracer amino acid dissolved in the culture medium is injected with a hypodermic syringe into a suspension of growing cells, giving complete mixing within 1 or 2 seconds. Samples are withdrawn with a small hypodermic syringe (arranged with a stop so that it delivers a constant sample size) and squirted onto the filter or into TCA.

Figure 1 shows the results of an experiment measuring the uptake of C^{14} -proline. After a lag of less than 10 seconds the proline is taken up into the compounds of the TCA precipitate at a constant rate until the supply in the medium approaches exhaustion. The total quantity taken up into the cell rises rapidly at first and then parallels the uptake into the protein. The difference between these two curves measures the quantity of adsorbed proline. This quantity rises rapidly at first, then remains constant for a period, and finally decreases as the proline is transferred to the protein after the supply in the medium is exhausted. The concentration of TCA-soluble proline per milliliter of cells is 500–1,000 times the concentration of proline in the medium, showing that the proline is bound in a nondiffusible form.

The radioactive material extracted from the cells with TCA after a 1-minute exposure to C^{14} -proline in a similar experiment has been shown to be authentic proline by paper-chromatographic fingerprinting.¹ We have not yet succeeded

in removing the adsorbed proline from the cell and still preserving its association with other cellular components.

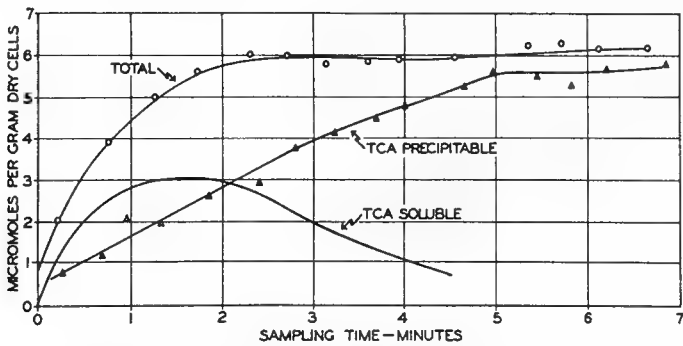


FIG. 1.—Incorporation of C¹⁴-proline by a suspension of growing *E. coli* cells. The temperature was 24° C. and the generation time about 2¼ hours. The suspension contained glucose, ammonia, mineral salts, 1(–)C¹⁴-proline at 1.2 × 10^{–6} molar and 0.2 mg. (dry weight) of cells per ml.

The cold TCA precipitate has been further fractionated,¹ and the results are shown in Table 1. The distribution among the fractions of the cold TCA precipitate is similar to that observed for other tracers which label cellular proteins. Chromatography of these fractions before hydrolysis shows no separation of the radioactivity from the proteins. Chromatography after hydrolysis shows that the radioactivity is almost entirely in proline, with traces in glutamic acid and arginine.

TABLE 1*
FRACTIONATION OF COLD TCA PRECIPITATE

Fraction	Per Cent of Radioactivity	Fraction	Per Cent of Radioactivity
Cold TCA precipitate	100	Hot TCA-soluble	5
Hot ethanol-soluble	15	Hot TCA precipitate	80

* In an experiment similar to that shown in Fig. 1 a sample of the suspension was injected into TCA at 1 minute. After 15 minutes the TCA precipitate was harvested and extracted with 80 per cent ethanol for one-half hour at 80° C. This precipitate was then extracted with 5 per cent TCA at 90° C. for one half hour, leaving nearly pure protein as the final precipitate.

The adsorption of proline is highly specific. Figure 2 shows the uptake of C¹⁴-proline under the same conditions as those in the experiment of Figure 1, except that fifteen other amino acids were added, each at 100 times the concentration of the proline. A proline contamination of 0.2 per cent in any of the other amino acids is sufficient to account for the slight difference between Figure 1 and Figure 2. If the adsorption of proline were not specific, the quantity adsorbed would have been reduced several hundredfold. When the proline is supplied at high concentrations (10^{–4} M), a greater adsorption (20 μM/gm dry) is observed. In this case the adsorption is reduced by the presence of other amino acids. It thus appears that there are two types of sites: specific sites which are the only ones involved

at the low concentrations used in most of these experiments and nonspecific sites which become prominent at higher concentrations.

The adsorption process also requires energy. Figure 3 shows the results of an experiment in which cells which had exhausted the supply of glucose several hours previously were supplied C^{14} -proline. The rate of adsorption is reduced by ap-

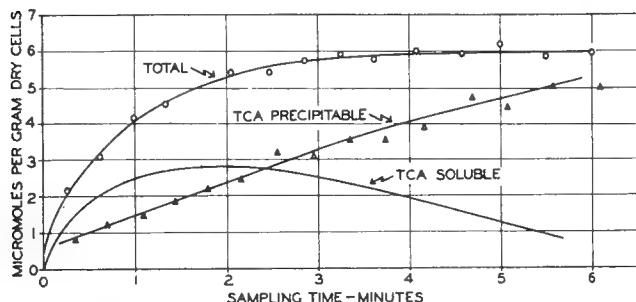


FIG. 2.—Incorporation of C^{14} -proline in the presence of other amino acids. The suspension was identical to that of Figure 1 with the addition of 0.013 mg per ml (about 10^{-4} molar) of each of the following: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine and valine.

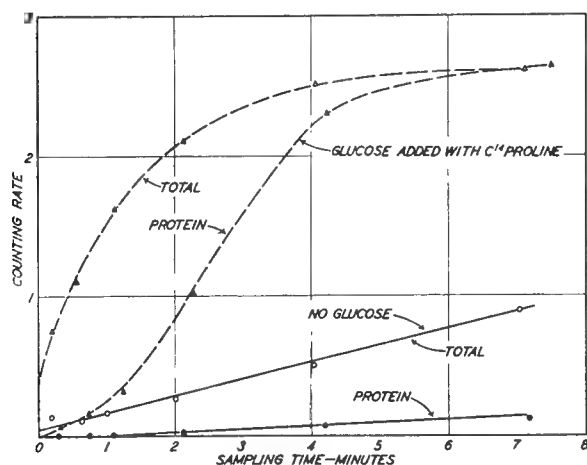


FIG. 3.—Effect of glucose on the incorporation of C^{14} -proline. Lower curves (solid line) show the incorporation in the absence of glucose. The upper curves (dotted line) show the incorporation when 0.1 per cent glucose was added with the C^{14} -proline. C^{14} proline concentration, 0.28×10^{-6} molar; cell concentration, 0.07 mg. (dry weight) per ml.; temperature, $37^\circ C$.

necessary for the amino acid to pass through the adsorbed pool to be utilized for protein synthesis or whether the adsorption is merely a storage mechanism.

To distinguish between these alternatives, a suspension of cells was pretreated for 1 minute in a solution of nonradioactive proline to build up within the cell a pool of unlabeled proline; then labeled proline was added and a series of samples withdrawn.

Figure 4 shows that there is a delay in the incorporation into the protein. The

proximately a factor of 20. This residual rate is probably due to endogenous reserves of energy which slowly become available. When glucose is added along with the C^{14} -proline, adsorption begins instantly and the protein synthesis seems to be delayed for less than a minute.

On the other hand, adsorption can proceed under certain conditions in which protein synthesis is blocked.

A methionine-requiring mutant rapidly adsorbed proline when growth was prevented by the absence of methionine. The adsorbed proline was not transferred to the protein until methionine was added to the medium. Similarly, nitrogen-deficient cells or cells treated with chloramphenicol ($50 \mu g/ml$) could adsorb proline even though its incorporation into protein was blocked.

The experiments described above show that the adsorbed materials pass on into the protein and that the adsorption is a specific process suggesting sites involved in protein synthesis.

They do not tell whether it is

shape of the incorporation curve agrees remarkably well with a curve calculated on the assumption that the slope of the protein-incorporation curve is proportional to the radioactivity of the adsorbed pool. Since the cells were pretreated with unlabeled proline, the total amount of proline in the adsorbed state should be approximately constant until the exogenous proline approaches exhaustion. Thus, if the adsorbed proline is the only source of proline for the protein and supplies proline at a constant rate, the rate of entry of radioactive proline into the protein will be proportional to the radioactivity of the adsorbed pool. If even a few per cent of the proline entering the protein had by-passed the adsorbed state, it would have caused a detectable initial rise in the protein-incorporation curve. Thus it is clear that the adsorption process is a necessary step in the incorporation of exogenous proline into the protein.

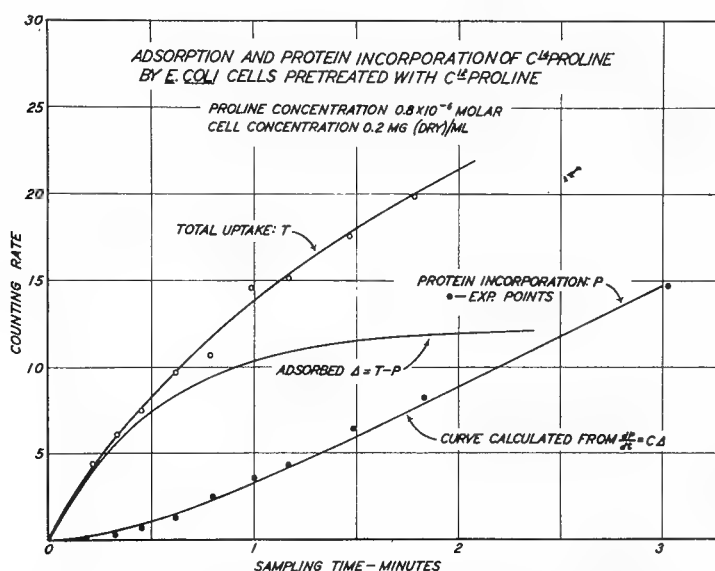


FIG. 4.— C^{12} -proline (0.8×10^{-6} molar) was added 1 minute before the carrier-free C^{14} -proline. An amount of medium was added with the C^{14} -proline so that there was no change in proline concentration.

At 0° C. the adsorption is negligible, as might be expected in an energy-requiring process. However, exchange can occur. Growing cells were suspended in unlabeled proline for 2 minutes at 24° C. and then chilled to 0° C., the chilling process taking about 5 minutes. C^{14} -proline was then added to the system and a series of samples taken. Figure 5 shows that the labeled proline entered the TCA-soluble fraction of the cell, but almost no incorporation into the TCA precipitate occurred. It appears that the external C^{14} -proline exchanged with the C^{12} -proline that was previously adsorbed at 24° C. and had remained on the sites during the chilling process. To show that exchange was occurring, a small amount of C^{12} -proline was added after equilibrium had been approached. The amount of C^{14} -proline adsorbed then fell as a new exchange equilibrium was approached. Specificity for proline adsorption was again shown, since the equilibrium was not displaced by the addition of a hundredfold excess of each of fifteen other amino acids.

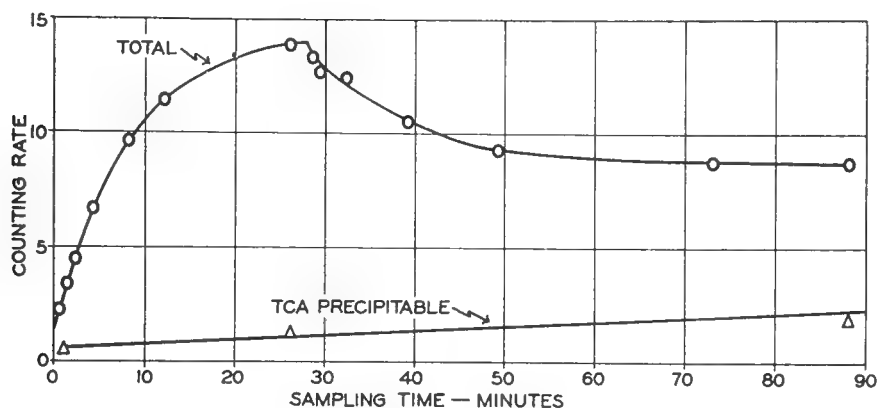


Fig. 5—Exchange between adsorbed and exogenous proline at 0° C. At 28 minutes C^{12} -proline was added. Circles represent total incorporation, triangles incorporation into TCA precipitate.

In order to study the stability of the binding of the proline, a suspension was prepared as above. After exchange equilibrium at 0° C. had been achieved, the cells were exposed to various reagents at 0° C. The data of Table 2 show that the adsorbed proline is freed from the cells by mild treatment—small shifts in pH or moderate concentrations of ethanol. It is indicated that the proline is not held by stable chemical bonds.

TABLE 2*
EXTRACTION OF ADSORBED PROLINE

Added Reagent	Final Concentration	Per Cent of TCA-soluble Proline Extracted
TCA	5%	100
TCA	0.25%	20
Ethanol	10%	0
Ethanol	20%	47
Ethanol	30%	95
Ethanol	40%	107
Butanol	10%	90
Toluene	Saturated	20
Pyridine	1%	0
Roccal	0.5%	100
Dinitrophenol	0.002 M	40
Glucose	10%	0
NaCl	10%	35
NaOH	pH 10.5	116
NaOH	pH 8.1	40
NaOH	pH 7.7	10
HCl	pH 6.5	0
HCl	pH 5.5	26
HCl	pH 4.7	50
HCl	pH 4.3	60
HCl	pH 2.8	47
HCl	pH 1.8	101
HCl	pH 1.0	100
Chill to -80° C. and thaw	{ Once	25
	{ Twice	37
Sonic disintegration to reduce optical density at 650 mμ by 70 per cent		80

* Samples of a suspension in exchange equilibrium were added to tubes at 0° C. containing reagents in the proper amounts to bring the final suspension to the condition described in the second column. After 10 minutes these suspensions were filtered, and the fraction of the TCA-soluble proline that had been extracted was calculated from the radioactivity of the precipitate.

These studies have been conducted chiefly with proline, but the principal points have also been checked with methionine. These two amino acids are particularly suited, as they are end products rather than intermediates of amino acid synthesis and are not extensively degraded by the cells. Furthermore, they are not present in growing cells (in the adsorbed state) in any appreciable quantity unless they are added as supplements to the medium. Some observations have been made using other amino acids, including glutamic acid, alanine, valine, tyrosine, phenylalanine, arginine, and lysine. In all cases the adsorption can be observed and distinguished from incorporation into proteins by kinetic measurements of the type described above. In addition, mixtures of fifteen labeled amino acids have been added and chromatograms made of the TCA-soluble fractions of successive samples. Those amino acids which are normally present in appreciable quantities when the cells grow in the glucose-ammonia medium (glutamic acid, alanine, and valine) show radioactivity in the TCA-soluble fraction for some time, whereas the radioactivity of the others is more rapidly incorporated into the protein.

No radioactive compounds in addition to the amino acids supplied were observed in the TCA-soluble fraction. These conditions would be very favorable for the detection of small peptides. The combination of the high specific radioactivity with short periods of observation forms what might be termed a "chemical microscope" which can focus on the newly incorporated material and ignore the much larger quantities of the other material already present in the cell. Neither were any transient intermediates found in the various fractions of the cold TCA-insoluble material. Accordingly, we find no evidence for peptides as intermediates of protein synthesis.

As shown above, the adsorption of proline and methionine is highly specific and requires energy. If the amino acids were converted into an "active" form, receiving energy from a common source before being adsorbed, it would be expected that an excess of other amino acids would compete for the energy source and would thereby interfere with the adsorption of proline and methionine. Since this does not occur, it seems reasonable to believe that the energy is utilized to prepare the site for subsequent adsorption of the amino acid. A part of the energy could remain in the amino acid site complex to supply energy for the synthesis of peptide bonds.

The kinetic studies show that this adsorption is a necessary step in the process of protein synthesis. However, adsorption is not necessarily followed by peptide-bond formation. No peptide fragments have been observed as transient intermediates. Neither was adsorbed proline bound into a peptide linkage in a mutant blocked by the lack of a single amino acid. A model of protein synthesis wherein the peptide bonds were formed in a process like knocking down a row of dominoes would be consistent with these observations.

We have not been able to determine what type of bond holds the amino acid; hydrogen bonding seems likely in view of the ease of extraction. Neither have we any indication as to what molecules provide the binding sites. Ribose nucleic acid is of course an attractive possibility in view of its long-known association with protein synthesis. The quantity of RNA in these cells is roughly 500 μ M of RNA nucleotides per gram of dry cells. Accordingly, if 5 per cent of the nucleotides provided binding sites for proline (proline makes up 5 mol per cent of the protein),

there should be 25 μM of sites available. This is more than adequate to account for the 3–5 μM of specific binding observed.

The formulation of a general theory of amino acid incorporation into proteins on the basis of these observations with proline and methionine would not be justified. However, any model of protein synthesis must provide a means of selectively locating amino acids and must provide the energy to form the peptide bonds. These studies of proline and methionine incorporation give an experimental demonstration of the operation of these two important processes in the synthesis of protein.

* Note added in proof: A report of similar studies carried out nearly simultaneously by G. N. Cohen and H. V. Rickenburg appears in *Comptes rendus des seances de l'Academie des sciences*, **240**, 2086.

¹ Various technical details such as culture media, chromatographic solvents, special chromatographic methods, and procedures for chemical fractionation of the cells are described in Roberts, Abelson, Cowie, Bolton, and Britten, "Studies of Biosynthesis in *Escherichia coli*," *Carnegie Inst. Washington Publ.*, No. 607, 1955.

Comment. This paper marked the beginning of a long series of investigations of amino acid pools both in *E. coli* and in yeast. It introduced the filter technique for rapid sampling and ended our interest in peptides as intermediates in protein synthesis.

Further work showed that the quantities of amino acids held in the cells was far too great to be attributed solely to adsorption on protein-forming templates. Thus the hope that these templates could be studied by measurements of properties of the pool soon faded. According to current ideas the templates constitute only a very small fraction of the RNA. Thus the maximum quantity that could be adsorbed to templates is much less than the observed pool.

Richard B. Roberts.

II.B.2 The Amino Acid Pool in Escherichia coli

(Reprinted, by permission, from Bacteriological Reviews, vol. 26, no. 3, pp. 292-335, September 1962.)

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I. INTRODUCTION

"For an angel went down at a certain season into the pool and troubled the water."

John 5:4

Bacteria maintain internally synthesized small molecules at high internal concentrations and in addition have the capacity to concentrate many compounds from the environment. Since the majority of these compounds are intermediates in synthesis, they are collectively termed the pool of metabolic intermediates or, simply, the "pool." However, the state of organization and ultimate chemical fate of exogenous compounds concentrated by the cell may be different from those of identical compounds synthesized by the cell.

Since the mechanism by which high internal concentrations are maintained is not understood

and the processes are obviously complex, it appears fruitless to enter into an extended discussion of the meaning of the term "pool." Therefore, we will simply define the "pool" as the total quantity of low molecular weight compounds that may be extracted from the cell under conditions such that the macromolecules are not degraded into low molecular weight subunits, for example, brief exposure to 5% trichloroacetic acid at room temperature.

Experiments have in general been designed to answer the following three questions: What are the mechanisms by which exogenous compounds are concentrated? What states of organization exist for compounds in the pool? What is the relationship of the pool to the mechanism of macromolecular synthesis?

At the present time unequivocal answers do

not exist for any of these three questions. However, the large body of experimental evidence does provide a restrictive set of conditions which theoretical models must satisfy, and supplies a background for the formulation of more refined questions.

The present paper is a description of several years of experimental work which in general has only been briefly described in print (2-4, 6). The implications of the evidence for the mechanism of pool formation are discussed in relation to several possible models. A mathematical analysis of the implications of the models is given in an appendix.

II. PRINCIPAL FEATURES OF POOL FORMATION AND MAINTENANCE

A. Introductory Discussion

Pool formation is an expression of the ability of the cell to obtain nutrients present at very low concentrations in the environment and to supply them to the synthetic machinery at high concentrations. This, perhaps, allows significant simplification of subsequent steps in macromolecular synthesis. One of the principal questions is whether the internally concentrated substances are free in solution within the cell or held in a more complex fashion. If the pool is simply a concentrated solution that pervades the cell, then the synthetically active structures within the cell are bathed in this solution, which is thus the "medium" in which synthesis occurs. On the other hand, the amino acids of the pool may be more closely associated with the substructures of the cell responsible for protein synthesis. They simply might be trapped in such substructures or they might be bound to them by labile chemical bonds. In the latter case it would be highly important to know the nature of the binding sites and how intimately they are related to the synthetic activities.

Since there is a large body of experimental evidence presented in this section, it seems well, for purposes of clarity, to summarize in advance the principal features of the pool that have been demonstrated:

1) Passage through the pool appears to be an obligate step for incorporation of an exogenous amino acid into protein.

2) Amino acids present in the pool are incorporated into protein at random, regardless of the length of time they have been in the pool.

3) Peptides do not appear to be intermediates in protein synthesis.

4) An energy source (such as glucose) is required for pool formation to occur at normal rates but is not required for maintenance of the pool for relatively long periods.

5) Specific pool formation mechanisms exist for each amino acid or group of structurally similar amino acids.

6) For any given amino acid there appears to be maximum pool size (or saturation value) at large external concentrations.

7) Any damage to the cells' integrity, or even mild treatments (for a bacterial cell), such as osmotic shock, leads to loss of the pool.

8) Exchange between pool and external amino acids occurs at a high rate, not only when there is steady flow through the pool but also in absence of glucose or at 0°C when the flow through the pool is strongly suppressed (conditions which also suppress pool formation).

B. A Typical Experiment

The experiments were performed with uniformly C^{14} -labeled amino acids of very high specific radioactivity, chromatographically prepared from *Chlorella* protein hydrolyzates (14, p. 47). The amino acids were chromatographically pure, and their purity was further checked by the suppression of the incorporation by *Escherichia coli* of a given labeled amino acid when pure amino acid carrier was present.

For low amino acid concentrations, constant pool sizes are established within 1 min. As a result, a technique had to be developed for sampling at 5- to 10-sec intervals in order to measure the kinetics of pool formation (6).

In a typical experiment, a suspension of *E. coli* strain B (ATCC 11303) was aerated in an open beaker in a temperature-controlled water bath. At the start of the experiment, the tracer was injected with a hypodermic syringe, in a moderate volume of medium, to give instantaneous mixing. Samples were withdrawn with a hypodermic syringe fitted with a stop to deliver a reproducible volume. These samples were either immediately filtered on a collodion membrane or injected into an equal volume of 10% trichloroacetic acid. The radioactivity of the cells collected on the filter measured the total incorporation, that is, the sum of the amount of labeled amino acid in the pool and the amount incorporated into protein. After about 10 min, the sample that was diluted into trichloroacetic acid was filtered on a similar collodion membrane. As the

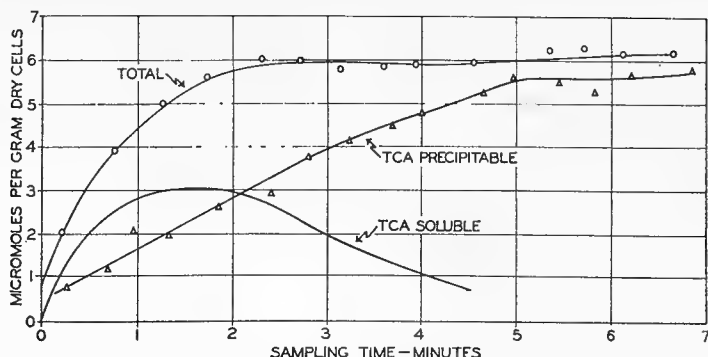


FIG. 1. Incorporation of C^{14} -proline by a suspension of growing *E. coli* cells. The temperature was $24^\circ C$ and the generation time, about $2\frac{1}{2}$ hr. The suspension contained glucose, ammonia, mineral salts, $1(-) C^{14}$ -proline at $1.2 \times 10^{-6} M$, and 0.2 mg, dry weight, of cells per ml (equal to 0.8 mg, wet weight, per ml). TCA = trichloroacetic acid.

pool was extracted by the 5% trichloroacetic acid, the radioactivity of this filter measured the incorporation into macromolecules. Most of the amino acids are utilized only for protein synthesis. For these amino acids, the radioactivity of the trichloroacetic acid precipitate is a direct measure of the incorporation into protein.

To avoid curling, the wet filters were cemented to plastic planchettes with rubber cement. The very thin, flat, and uniform layers of cells on the collodion membranes gave precisely reproducible counting rates. Thus the pool radioactivity could be accurately calculated by subtracting the quantity incorporated into protein from the total.

Figure 1 shows the results of an experiment measuring the uptake of C^{14} -proline. After a lag of less than 10 sec, the proline is taken up into the compounds of the trichloroacetic acid precipitate at a constant rate until the supply in the medium approaches exhaustion. The total quantity taken up into the cell rises rapidly at first and then parallels the uptake into the protein, until the external amino acid is almost exhausted. The difference between these two curves measures the quantity of proline in the pool. This quantity rises rapidly at first, then remains constant for a period, and finally decreases as the proline is transferred to the protein after the supply in the medium is exhausted. The maximum concentration of trichloroacetic acid-soluble proline per milliliter of cells is 600 times the initial concentration of proline in the medium.

Before the supplemental proline is added, and after it is exhausted, the cell internally synthesizes proline as required for protein. In this ex-

periment the supplemental proline supplies, at maximum, half of this requirement, while at higher concentrations internal synthesis is almost completely suppressed (see Appendix). Such a suppression, however, does not occur for all amino acid supplements (14, p. 196).

The radioactive material extracted from the cells with trichloroacetic acid after a 1-min exposure to C^{14} -proline, in a similar experiment, has been shown to be authentic proline by paper chromatographic fingerprinting (14, p. 191). When relatively high concentrations ($10^{-4} M$) of proline are supplied, however, the cells convert some of the proline to arginine and glutamic acid after a delay of about $\frac{1}{2}$ hr. In such an experiment, the rate of incorporation of radioactivity into protein increases appreciably after $\frac{1}{2}$ hr, and chromatography shows the presence of the other amino acids in the pool. This is an interesting example of the induced reversal of reaction sequences which are normally entirely unidirectional.

The experiments with C^{14} -proline described below have been performed under conditions in which conversion to other amino acids is negligible. For experiments with other amino acids, such as valine, which is rapidly converted to leucine, chromatography has been used to check the results, and proper allowance has been made for conversions or degradations occurring in the pool.

C. Passage through Pool Obligate for Entry into Protein

The experiment illustrated in Fig. 1 shows that the amino acids of the pool are readily availa-

ble for protein synthesis. It is not immediately clear, however, that entry into the pool is a necessary step in protein synthesis. Two possible interpretations are shown in Fig. 2. The lower diagram shows the incorporation curve to be expected if the externally added labeled amino acid must mix with a pre-existing unlabeled pool before entering the protein. The rate of entry of radioactivity into protein is initially zero.

On the other hand, if the labeled amino acid by-passes the pool, it will initially enter the protein at a rate determined by the relative by-pass flow. The upper diagram on Fig. 2 is for an ex-

treme case in which the by-pass flow is large and the quantity of amino acid pre-existing in the pool is so great that the specific radioactivity of the added amino acid is reduced significantly by dilution. Examples of large by-pass flows occur in the incorporation of nucleic acid bases into ribonucleic acid (11).

In order to assess the possible existence of a by-pass around the proline pool, an experiment was performed in which C^{12} -proline was first added to establish a pool of unlabeled amino acid. After 1 min, a small quantity of C^{14} -proline of high specific radioactivity was added without appreciably altering the proline concentration or the steady pool size.

Figure 3 shows the results of such an experiment. As the amount of proline in the pool is constant, the specific radioactivity of the pool is simply proportional to the measured total radioactivity of the pool (P in Fig. 3). Since the rate of entry of proline into protein is also constant, the rate of entry of C^{14} -proline into the protein will be proportional to the radioactivity of the pool, if the pool is the source of proline for protein synthesis. The shape of the measured curve for incorporation of label into protein agrees remarkably well with the curve calculated on this assumption.

If even a few per cent of the proline entering the protein had by-passed the pool, as suggested by the upper drawing in Fig. 2, it would have

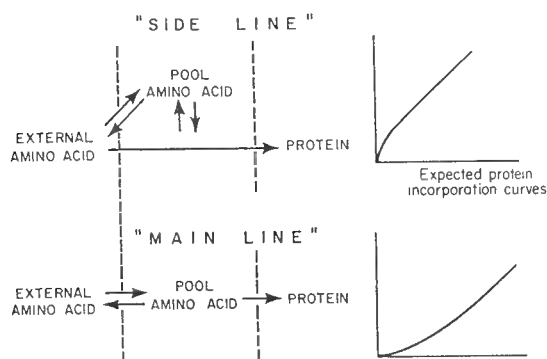


FIG. 2. Schematic illustration of two interpretations of the function of the pool. At the right are shown the expected curves for incorporation of C^{14} -proline into the protein when the cells have been pretreated with C^{12} -proline.

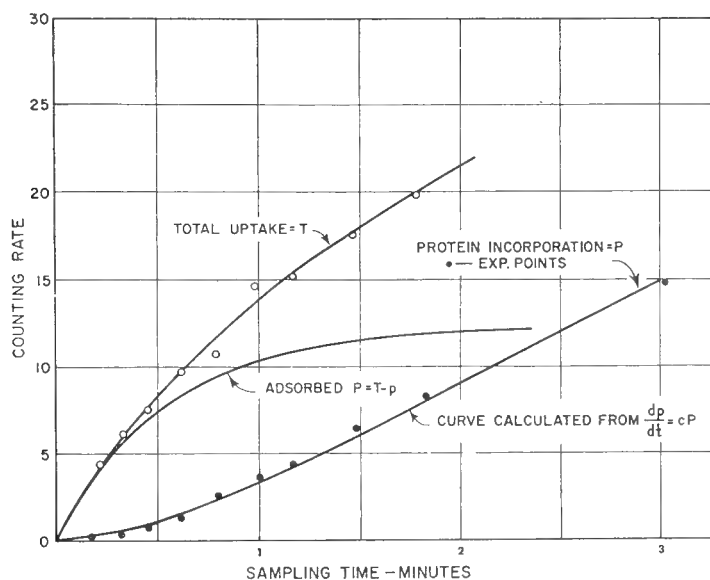


FIG. 3. C^{12} -proline ($0.8 \times 10^{-6} M$) was added 1 min before the carrier-free C^{14} -proline. An amount of medium was added with the C^{14} -proline such that there was no change in proline concentration.

caused a detectable initial rise in the protein incorporation curve. This experiment demonstrates that passage through the pool is an obligatory step in the incorporation of exogenous proline into the protein. The precision with which the experimental data fit the calculated curve also shows that pool amino acid is utilized for protein synthesis at random. The selection of an amino acid molecule is independent of the length of time the amino acid has been in the pool. If the amino acid previously existing in the pool had an advantage in this respect, there would be a further delay of incorporation of tracer into the protein.

A similar experiment has been performed with C^{14} -valine with identical results (Fig. 12). While no other amino acids have been tested to this degree of precision, the lack of conflicting evidence from many experiments with other amino acids indicates that this is a valid general conclusion for *E. coli*.

It cannot, of course, be concluded from such an experiment that internally synthesized amino acids do not by-pass the pool to some extent. That such a by-pass may operate is suggested by the failure of lysine and aspartic acid, even at very high external concentrations, to substitute completely for the internally synthesized amino acid in protein synthesis (14). A similar phenomenon occurs in the case of citric acid (14, p. 199). The definite proof of such an internal by-pass would be worth while, since it would imply that the pool of amino acid concentrated from the medium is organized within the cell in a way different from at least part of the pool of internally synthesized amino acid.

D. Failure to Observe Peptide Intermediates

When small quantities of amino acids of high specific radioactivity (*Chlorella* protein hydrol- yzate containing 10% C^{14}) are supplied to the cells, very rapid uptake into the pool is observed. Chromatographic analysis of trichloroacetic acid or alcoholic extracts of samples taken at intervals indicates that certain amino acids (those with small native pools) are very rapidly incorporated into the protein and that others, such as glutamic acid, are completely incorporated into protein only after 10 to 15 min. The chromatograms do not show significant quantities of radioactive compounds other than the amino acids supplied.

In another type of experiment, cells that had exhausted their supply of glucose were given a small quantity of C^{14} -glucose (uniformly labeled) of very high specific radioactivity. Under these conditions, about 30% of the C^{14} incorporated is incorporated into macromolecules and 70% remains in the trichloroacetic acid-extractable pool. (A further discussion of this experiment appears below.) Chromatography of this pool shows that the principal part of the radioactivity occurs in the usual pool amino acids that occur in a growing cell. In addition, small quantities of glutamine and asparagine have been identified. Traces of several unidentified compounds are present, but these do not appear to be peptides.

The sensitivity of these experiments to intermediates present in trace quantities is high. For a number of the amino acids, the total quantity present in the pool (tracer plus native amino acid) corresponds to the amount utilized for protein synthesis in about 30 sec. Peptides containing these amino acids would be detected if the quantity corresponded to only a 1-sec requirement for protein synthesis.

It should be pointed out that these experiments do not eliminate the possibility of the occurrence of small peptides as intermediates in protein synthesis. Rather, they demonstrate that the trichloroacetic acid-soluble pool of these compounds is extremely small, if it exists at all. That such pools of peptides are very small or absent is also indicated by the rapidity of the incorporation of $S^{35}O_4$ into protein under conditions of sulfur starvation (12).

E. Requirement for Energy

Figure 4 shows the results of an experiment in which cells that had exhausted the supply of glucose several hours previously were supplied C^{14} -proline. The rate of pool formation is reduced by approximately a factor of 20. This residual rate is probably due to endogenous reserves of energy which slowly become available. When glucose is added along with the C^{14} -proline, pool formation begins instantly and protein synthesis is delayed for less than 1 min. Studies of pools formed in the presence of other amino acids indicate that the requirement for glucose (or some equivalent energy source) is quite general. At low concentrations, however, some of the other amino acids seem to be taken up to a greater extent than proline, in the absence of glucose.

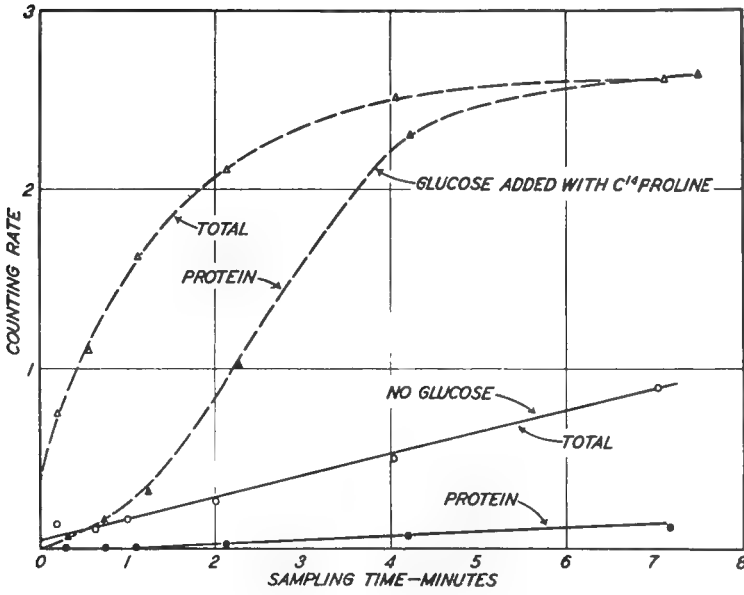


FIG. 4. Effect of glucose on the incorporation of C^{14} -proline. Lower curves (solid line) show the incorporation in the absence of glucose. The upper curves (dashed line) show the incorporation when 0.1% glucose was added with the C^{14} -proline. C^{14} -proline concentration, 0.28×10^{-6} M; cell concentration, 0.07 mg, dry weight, per ml; temperature, 37 C.

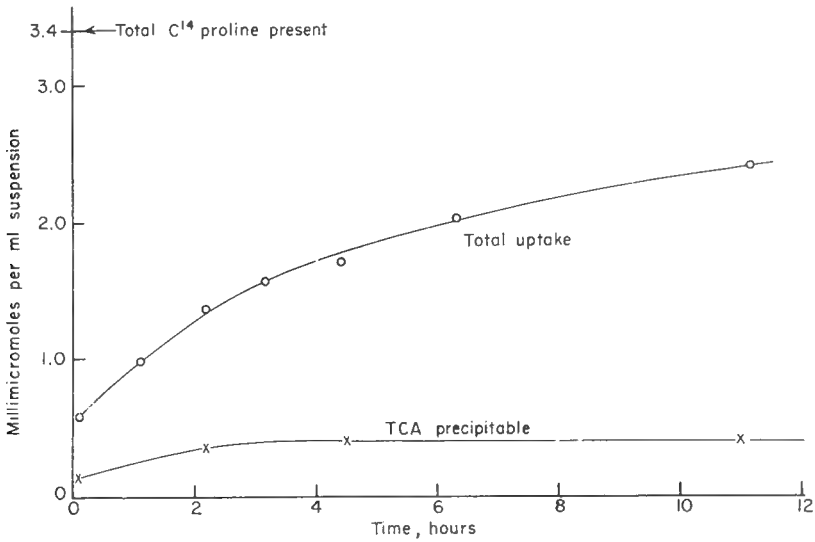


FIG. 5. Proline pool formation at 0 C, 1.1 mg of wet cells per ml of suspension; 3.4×10^{-6} M C^{14} -proline. Exponentially growing cells were chilled to 0 C for 45 min before C^{14} -proline was added. TCA = trichloroacetic acid.

Figure 5 shows the kinetics of proline pool formation at 0 C. Note that the abscissa scale is in hours, not minutes. As might be expected in an energy-requiring process, the rate of pool formation is very low. Both in the absence of glu-

cose and at 0 C, pools are formed very slowly, but pre-existing pools are maintained for long periods of time. Further experiments on the loss and exchange of pools under these conditions are described below.

The concentration of the pool amino acid, calculated over the whole cell volume, may be several thousand times the external concentration. Some source of energy is necessary to establish such a concentration difference. It could, however, come immediately from reactions coupled to glycolysis or have been stored previously in sites for adsorption of the amino acid. The former alternative is supported by the requirement for glucose. The fact that endogenous reserves do not in general supply the small amount of energy required, of course, could not have been predicted.

While pools are formed very slowly in the absence of glucose or at 0 C, preformed pools, if they are not too large, are nevertheless maintained for long periods of time. An experiment described below (Fig. 15) shows the effect of the exhaustion of glucose after formation of a proline pool. In this case, the pool was maintained for 20 min without change. Other experiments show that such pools are maintained for many hours. At 0 C, pools that have been formed before the cells have been chilled are maintained for days.

It has been found that when a very large pool (near saturation) is formed at 25 C, a considerable fraction of the pool is lost at the time of chilling to 0 C. The remaining part of the pool, however, appears to be stable for long periods. The stability of very large pools in the absence of glucose at 25 C has not been tested.

In one type of experiment, growing cells were allowed to exhaust the glucose supply, and then C^{14} -glucose of very high specific radioactivity was added in an amount sufficient to support growth for only a few minutes. Seventy per cent of the incorporated radioactivity quickly appeared in the trichloroacetic acid-soluble fraction and remained there without significant change for several hours. After $\frac{1}{2}$ hr, samples of the trichloroacetic acid-soluble pool and the medium were withdrawn and analyzed by chromatography. Large quantities of amino acids were found in the pool and traces in the medium. The ratios of the concentrations of the amino acids in the cells to the concentrations in the medium were evaluated; they ranged from 28,000 for valine, 14,000 for glutamate, and 7,300 for proline to 2,300 for aspartic acid. Again, it is clearly shown that the cell has the capacity in the absence of glucose to maintain a highly concentrated metabolic pool.

F. Lability of the Pool

A large part of the pool is lost when the cell is damaged in almost any fashion. Mild treatments which do not interfere with subsequent growth of the cell may cause the pool to be lost completely. On the other hand, the deprivation of most nutrients does not cause the pool to be lost. Pools may be formed under a variety of conditions that block synthesis (such as nitrogen starvation or the presence of chloramphenicol) so long as an energy source is present.

Table 1 presents the results of a study of the stability of the proline pool in the presence of various reagents at 0 C. Cells containing an unlabeled proline pool were chilled to 0 C, and the pool was labeled by exchange as described in the section on zero-degree exchange. Samples of these cells were then treated and assayed as described in the legend to the table. The data in this table show that the pool is released from the cells by mild treatment from a chemical point of view, such as small shifts in pH or moderate concentrations of ethanol. If chemical bonds are involved in holding the pool, they are extremely labile.

Physical damage to the cells also releases the pool. As shown in Table 1, freezing and thawing release part of the pool, although the cells grow with little lag after this treatment. A water wash will remove the pool entirely and, after this treatment, the cells will grow normally within 2 min after restoration to the normal medium. Details of the effects of osmotic shock are described below. Any of the methods that have been used to disrupt the cells also cause the pool to be released. Examination by chromatography shows no sign of chemical modification of amino acids that have been concentrated in the pool, except for conversions occurring in the normal pathways of amino acid synthesis. Only traces of pool amino acids have been found in association with macromolecules after disruption of the cells.

G. Specificity of Pool Formation

Small proline pools are entirely uninfluenced by other amino acids at relatively high concentrations. Figure 6 shows the uptake of C^{14} -proline under the same conditions as those for the experiment shown in Fig. 1, except that 15 other amino acids were added, each at 100 times the concentration of the proline. The pool size and initial rate of pool formation are identical with

TABLE 1. *Extraction of proline pool at 0 C^a*

Added reagent or procedure	Final concentration or pH	Trichloroacetic acid-soluble proline extracted
		%
Trichloroacetic acid	5%	100
Trichloroacetic acid	0.25%	20
Ethanol	10%	0
Ethanol	20%	47
Ethanol	30%	95
Ethanol	40%	107
Butanol	10%	90
Toluene	Saturated	20
Pyridine	1%	0
Roccal	0.5%	100
Dinitrophenol	0.002 M	40
Glucose	10%	0
NaCl	10%	35
NaOH	pH 10.5	116
NaOH	pH 8.1	40
NaOH	pH 7.7	10
HCl	pH 6.5	0
HCl	pH 5.5	26
HCl	pH 4.7	50
HCl	pH 4.3	60
HCl	pH 2.8	47
HCl	pH 1.8	101
HCl	pH 1.0	100
Chill to -80 C and thaw	Once	25
	Twice	37
Sonic disintegration to reduce optical density at 650 m μ by 70%		80

^a Samples of a suspension in exchange equilibrium were added to tubes at 0 C containing reagents in the proper amounts to bring the final suspension to the condition described in the second column. After 10 min these suspensions were filtered, and the fraction of the trichloroacetic acid-soluble proline that had been extracted was calculated from the radioactivity of the precipitate.

those shown in Fig. 1. The time required for completion of the incorporation into protein is extended by about 30%. This difference is probably due to the presence of proline impurity (to the extent of 0.02%) in the mixture of other amino acids.

This result clearly demonstrates a high degree of specificity. It must be pointed out, however, that the proline pool size and rate of formation

increase with the external concentration at this low concentration (see below). As a result, carrier proline itself would have to be added at several times the concentration of the C¹⁴-proline in order to reduce appreciably the amount of C¹⁴-proline appearing in the pool at any time. This situation merely reduces the sensitivity of the test for certain types of interference by other amino acids. Since the other amino acids were present at 100 times the proline concentration, it may still be concluded that they have very little affinity for the specific mechanisms for proline pool formation.

The formation of very large proline pools is, however, interfered with by other amino acids. At high concentrations of proline, the proline pool saturates at a value of about 240 μ moles per g, dry weight. In the presence of high concentrations of other amino acids, the maximal proline pool is reduced by a large factor. A similar conclusion was reached from quite a different type of experiment. The maximal pool size for proline rises to 1,000 μ moles per g of dry cells in media of high osmotic strength (see discussion of osmotic properties of the pool, below). Under the same conditions, however, the total pool for all amino acids formed from casein hydrolyzate (20 mg per ml) was measured to be only 1,000 μ moles per g, dry weight.

It thus appears that there are specific mechanisms for the formation and maintenance of small amino acid pools and less specific, or completely nonspecific, mechanisms for the formation of very large pools. It would be possible to measure the maximal size of the specific and nonspecific pools by examining in detail the influence of other amino acids as a function of concentration, but this work has not been carried out.

Whereas there appears to be a concentrating system which is entirely specific for proline, there are other concentrating systems which apparently function for groups of similar amino acids (7, 8). Our observations on the interactions among one of these groups (valine, leucine, and isoleucine) will be discussed in the following paragraphs.

When carrier-free valine is supplied, the cells almost entirely remove it from the medium within about 10 sec, as shown by the upper curves in Fig. 7. The label is also very rapidly incorporated into protein. The effect of a moderate concentration of isoleucine (2.3×10^{-6} M) is

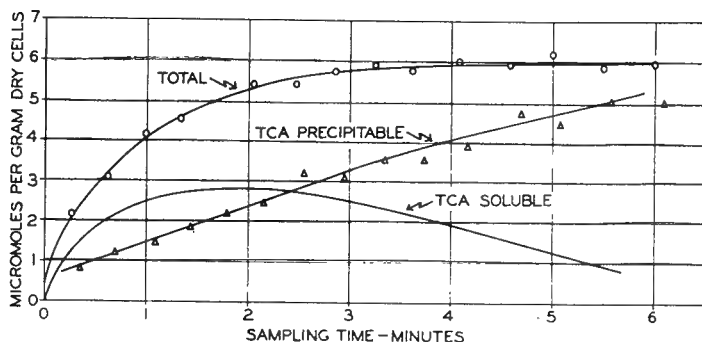


FIG. 6. Incorporation of C^{14} -proline in the presence of other amino acids. The suspension was identical with that of Fig. 1 with the addition of 0.013 mg per ml (about 10^{-4} M) of each of the following: alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, and valine. TCA = trichloroacetic acid.

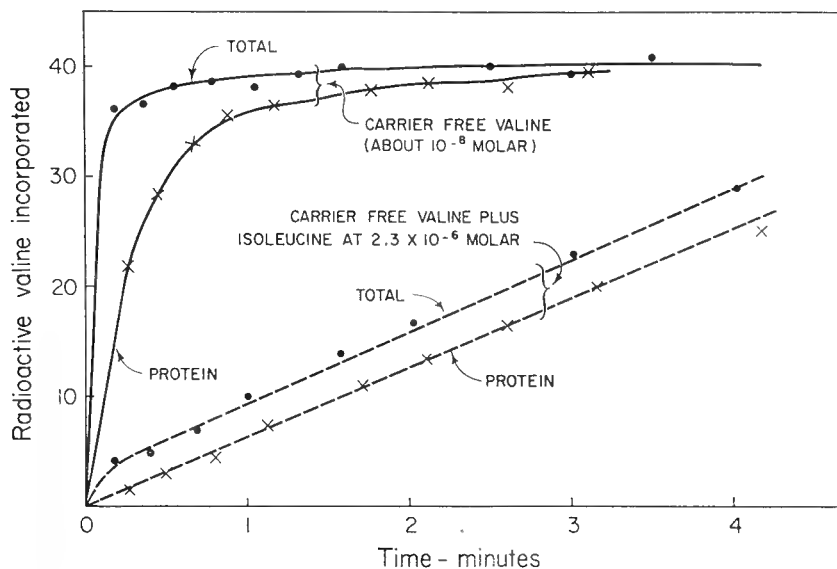


FIG. 7. Effect of isoleucine on the incorporation of valine at very low concentration: 0.125 mg per ml dry weight, of cells growing at 25 C. Upper curves (—), carrier-free C^{14} -valine, about 10^{-8} M. Lower curves (---), carrier-free valine plus C^{12} -isoleucine at 2.3×10^{-6} M.

shown in the lower curves. The isoleucine reduces the rate of incorporation of the valine into the cell by more than a factor of 50. In this case, the quantity of valine supplied is so small that it does not significantly alter the size of the previously existing valine pool. One simply observes the rate of entry of the tracer valine into the pool through the pool-forming mechanism and its entry into protein after dilution by internally synthesized valine in the pool. For both examples in Fig. 7, the label present in the pool is utilized at a rate such that it would be exhausted in about 30 sec if more were not flowing in. Thus, the

native valine pool equals 30 sec worth of valine requirement for protein, or 1.5 μ moles per g of dry cells.

The strong suppression of the rate of valine uptake by the isoleucine indicates that there is a common mechanism for the concentration of the two amino acids by the cell. In addition, an important feature of this concentrating mechanism is demonstrated by the strong interference at low concentrations where both amino acid pools are far below their saturation values. This result is most simply interpreted as an interference between the two amino acids at a cata-

TABLE 2. *Summary of the interactions of isoleucine, leucine, valine, and related compounds during pool formation^a*

Labeled compound	Concentration	Competitor	Concentration	Suppression of pool	Suppression in rate of incorporation into protein
	$\mu\text{g}/\text{m}$		$\mu\text{g}/\text{ml}$	%	%
1. Isoleucine	0.29	Leucine	8.8	98	75
2. Isoleucine	0.17	Leucine	9.1	98	82
3. Isoleucine	0.29	Valine	8.8	91	75
4. Isoleucine	0.17	Valine	9.1	94	75
5. Leucine	0.02	Valine	3.6	>60	
6. Leucine	0.29	Valine	10.0	86	10
7. Leucine	0.29	Valine	10.0	90	35
8. Leucine	0.29	Isoleucine	10.0	95	45
9. Leucine	0.32	Norleucine ^b	9.6	0	0
10. Leucine	0.32	Norvaline ^b	9.6	67	58
11. Valine	0.30	Leucine	9.1	80	73
12. Valine	0.007	Isoleucine	0.3	90	92
13. Valine	0.02	Isoleucine	10.0	94	>95
14. Valine	0.15	Isoleucine	0.075	16	20
15. Valine	0.15	Isoleucine	0.15	32	39
16. Valine	0.15	Isoleucine	0.30	54	54
17. Valine	0.15	Isoleucine	1.5	85	89
18. Valine	0.10	Isoleucine	4.0	87	90
19. Valine	0.30	Isoleucine	10.0	>90	>95
20. Valine	0.30	D-Valine	10.0	0	0
21. Valine	0.30	Norleucine ^b	10.0	65	0
22. Valine	0.30	Norvaline ^b	10.0	84	84
23. Valine	0.29	α -Ketoisovalerate	0.29	+18 ^c	30
24. Valine	0.29	α -Ketoisovalerate	0.87	+35 ^c	45
25. Valine	0.29	α -Ketoisovalerate	2.9	+65 ^c	58
26. Valine	0.30	α -Ketoisovalerate	7.5		65

^a The values for the suppression of the pool and reduction of the rate of incorporation of radioactivity into the protein are calculated from individual experiments such as those shown in Fig. 8.

^b Norvaline at 10 μg per ml suppresses growth rate by 42%; norleucine at 10 μg per ml does not suppress growth rate.

^c Increase in pool size.

lytic step in the pool-forming mechanism. In order for the strong interference to occur, however, this catalytic site must be nearly saturated with isoleucine at a concentration far below that at which saturation of the pool itself occurs for isoleucine. A similar conclusion can be drawn from the small variation in the rate of valine pool formation shown in Table 4.

Table 2 shows the results of a large number of experiments designed to explore the interactions in pool formation of valine, leucine, isoleucine, and a few related compounds. The data in this table were obtained from measurements of the kinetics of pool formation similar to those illustrated in Fig. 8. It is obvious that a common mechanism plays a part in the concentration of

these three amino acids by the cell. Norleucine and norvaline also have some affinity, but D-valine has no measurable affinity for this step in the concentration process. Apparently α -ketoisovalerate is also concentrated by the cells, but by a separate mechanism. The reduction of the rate of incorporation of C¹⁴-valine into protein (shown in the last column of Table 2) indicates that α -ketoisovalerate is converted into valine in the pool and thus dilutes the tracer. The last column in Table 2 shows the effect of the competing compounds on the rate of incorporation of label into protein. A reduction in this rate, except in the case just mentioned, results from a dilution in the pool of the C¹⁴-amino acid by internally synthesized C¹²-amino acid.

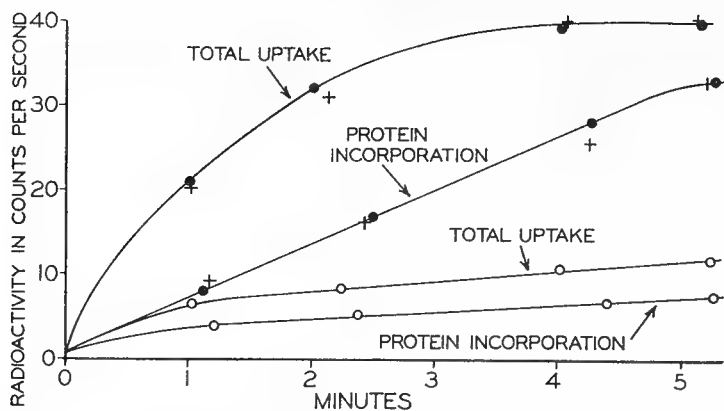


FIG. 8. Typical experiment from which the data of Table 2 were obtained. For all the curves, $0.3 \mu\text{g}$ per ml of C^{14} -valine was added to 0.08 mg , dry weight, per ml of growing cells at time zero. For the control (●), no competitor was present. For the curves marked (+), $10 \mu\text{g}$ per ml of D-valine were added at time zero. For the curves marked (○), $10 \mu\text{g}$ per ml of norvaline were added at time zero.

Table 2 shows that a simple reciprocity does not occur in the interaction of isoleucine and valine. As shown in lines 19 and 3 of Table 2, the effect of isoleucine on valine is greater than that of valine on isoleucine. Line 15, however, shows that an equal quantity of isoleucine depresses the valine pool by only 30%.

Isoleucine acts, in effect, as a "dog in the manger" in suppressing the valine pool. Line 16 of Table 2 shows that when isoleucine is present at twice the valine concentration, the valine pool is suppressed to one-half its normal value at this concentration. In this concentration range, however, if the valine concentration is tripled, the pool is tripled. Indeed, the maximum pool size for either valine or leucine is at least 10 times the pool size at the concentration used. Another example leading to a similar conclusion has been discussed above.

In another type of experiment, in order to show the displacement of the pool by a competitor, leucine was added after a C^{14} -isoleucine pool had been formed. Figure 9 shows the uptake of C^{14} -isoleucine as control. Figure 10 shows the results of an initially identical experiment in which C^{12} -isoleucine was added at 40 sec at 30 times the concentration of the tracer. With this concentration change, the pool does not increase in proportion to the concentration, and C^{14} -isoleucine is removed from the pool by exchange. The specific radioactivity of the pool isoleucine immediately starts to decrease, and as a result, the rate of incorporation of C^{14} -isoleucine into

the protein decreases to a steady-state value one-thirtieth of the control.

Figure 11 shows the results of the corresponding experiment in which an equally large quantity of C^{12} -leucine was added at 40 sec. The C^{14} -isoleucine is removed from the pool by exchange with the leucine. During the 40 sec after addition, while a measurable quantity of isoleucine remains in the pool, the rate of isoleucine incorporation into the protein is unaffected. After this period, when the isoleucine pool has dropped to a very small value, the incorporation into protein continues at about one-sixth the rate of the control. Similar experiments have been performed for various combinations of valine, leucine, and isoleucine. In each case, the rate of incorporation into protein of the labeled compound from the pool is unaffected until the quantity in the pool drops to a low value. The degree of suppression of the pool and rate of protein incorporation are different in the various cases.

In concluding the discussion of specificity of pool formation, it is worth while to point out that a large number of concentrating systems specific for given compounds or groups of compounds are now known in *E. coli*. In addition to the amino acid systems, several have been identified for sugars and nucleic acid bases. The number that are known at present probably does not exceed a dozen, but if all possible low molecular weight metabolites were tested, the number of specific transport mechanisms would probably turn out to be many times larger. This does not

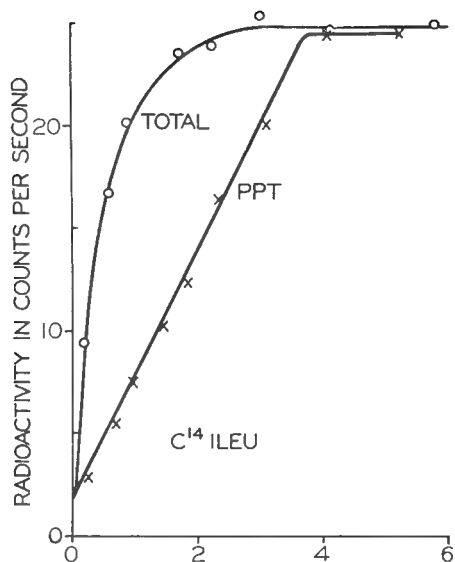


FIG. 9. Leucine-isoleucine interaction, control; $0.3 \mu\text{g}$ per ml of C^{14} -isoleucine was added at time zero to 0.25 mg , dry weight, per ml of growing cells.

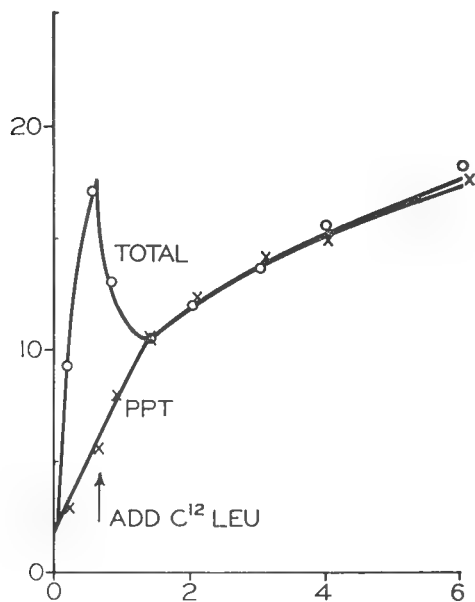


FIG. 11. Leucine-isoleucine interaction; effect of leucine competitor. Same as Fig. 9 with $10 \mu\text{g}$ per ml of C^{12} -leucine added at 40 sec.

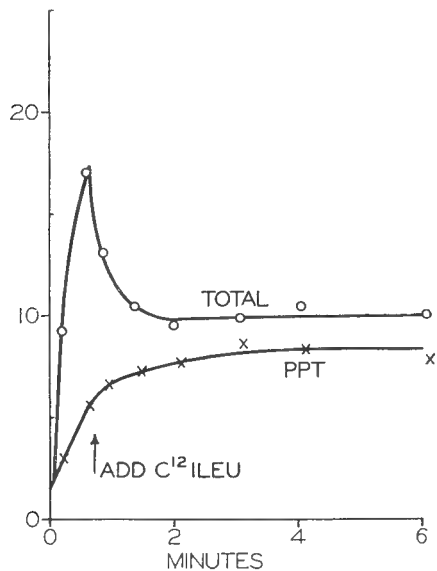


FIG. 10. Leucine-isoleucine interaction; effect of carrier isoleucine. Same as Fig. 9 with $10 \mu\text{g}$ per ml of C^{12} -isoleucine added at 40 sec.

add very much to the known number of enzymes in bacteria, but when it is considered that each of these is coupled to an energy-supplying system, it nevertheless becomes an impressive array. The implication is that concentrating systems have been important in the evolution of bacteria. Further, if they are all located in the cell mem-

brane, as has been suggested, and there are many sites for each function, the membrane is indeed a complex structure.

H. Exchange between the Pool and the Environment

In Fig. 12 are shown the results of an experiment performed to measure the rate of exchange when there is a steady flow of amino acid through the pool (see also Fig. 3). C^{12} -valine ($2.5 \times 10^{-6} \text{ M}$) was initially added to a growing culture of cells. Four minutes later, when the pool had reached a steady value, as shown by control experiments, C^{14} -valine was added without appreciably altering the external concentration of valine.

That this experiment demonstrates exchange between the pool valine and exogenous valine may be seen by referring to the two drawings in Fig. 13. The drawings represent the expected kinetics of labeling of the pool and protein when tracer is added to a system in which a constant-size pool has been previously established and where sufficient valine is present to maintain a constant pool for the duration of the experiment.

The lower drawing represents the case of no exchange. Molecules of labeled amino acid from the environment can only enter the pool as molecules leave the pool to enter protein. The total

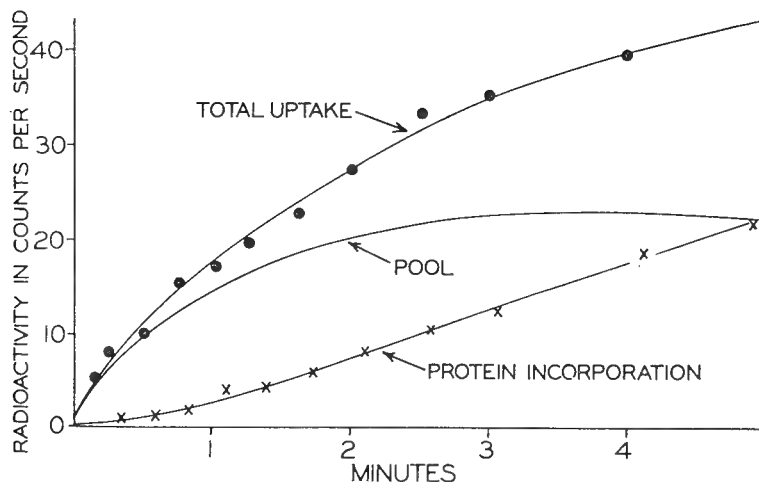


FIG. 12. Demonstration of exchange when a steady pool has been established. Growing cells (0.07 mg. dry weight, per ml) were supplied 0.3 μ g per ml of C^{12} -valine 4 min before the addition of the C^{14} -valine. At the time C^{14} -valine was added, the unlabeled pool had reached a steady state. The rapid initial incorporation of C^{14} -valine shows that exchange is occurring between pool valine and external valine.

label in the cell therefore rises linearly from the time of addition of the tracer. The specific radioactivity of the pool slowly rises as C^{14} -valine enters the pool, and the rate of entry of the label into protein rises in proportion to this specific radioactivity.

The upper drawing represents the expected kinetics of labeling when exchange occurs at a rate considerably greater than the rate of utilization for protein. In this case, labeled amino acid enters the pool initially at a high rate. Later, when the specific activity of pool amino acid equals that present externally, labeled amino acid enters the cell at just the rate at which it is utilized for protein synthesis. It is clear that the upper pair of curves is very similar to the pair shown in Fig. 12, and therefore it may be concluded that exchange is an important process for the entry of labeled valine into the pool. Observations with other amino acids indicate that this is a general phenomenon during pool formation in *E. coli*.

A simple calculation shows that the radioactivity of the pool valine should vary with time according to the relation, $P^* = P_E^* (1 - e^{-t/T})$, where P_E^* is the value after the specific activity of the pool valine equals that in the medium. The time constant, T , depends on the rate of incorporation of valine into protein and the rate of exchange between pool and external valine. The experimental curve of Fig. 12 fits this rela-

tion very accurately, and $T = 71$ sec. The time constant expected if there were no exchange would have been 241 sec.

The rate of exchange may be expressed more simply as shown in Fig. 14. The rate of entry of labeled amino acid into the pool is more than 3 times the net flow of amino acid through the pool.

An exogenous supply of energy (glucose) is not necessary for exchange to occur. Figure 15 gives the results of an experiment in which non-radioactive proline and a limited supply of glucose were added simultaneously to two identical cultures. C^{14} -proline was added to culture A at zero time and to culture B at 13 min. In both cultures the glucose was completely exhausted at 10 min. Experiment A (solid line) shows that the pool does not increase after the glucose is exhausted, but is maintained at a constant size. Protein synthesis also ceases at the time when glucose is exhausted. Experiment B (dashed line) shows, however, that exogenous proline enters the constant-size pool at a rapid rate. (The lack of entry of C^{14} -proline into the protein in B demonstrates that the glucose was indeed exhausted.) It is clear that exchange occurs between the proline in the pool and exogenous proline in the absence of an energy source. This experiment also suggests that the rate of exchange is not influenced by the presence of glucose. The experi-

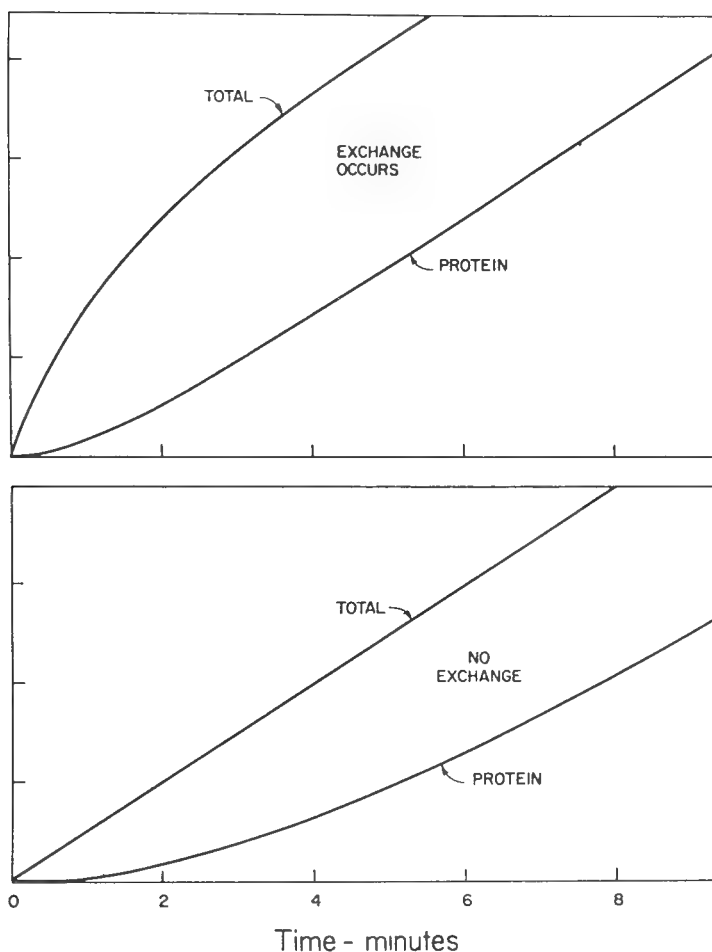


FIG. 13. Kinetics of incorporation of labeled amino acid into cells with a pre-existing pool. These curves were calculated for a pool size and rate of protein synthesis comparable to those of Fig. 12. It was assumed that no change in pool size occurred at the time the tracer was added or at later times. The lower pair of curves is for the case of no exchange. The upper pair of curves was calculated for an exchange rate comparable to that implied by Fig. 12. The radioactivity of the pool is the difference between the total and protein curves, in each case.

ments, however, have not been performed with sufficient accuracy to establish this point.

At 0 C, exchange occurs, while pool formation is very strongly suppressed. In order to study exchange at 0 C, a pool of the appropriate size must be formed before the suspension is chilled. Such pools have usually been formed with unlabeled amino acids so that radioactivity does not enter the protein. Figure 16 gives the results of an experiment in which growing cells were suspended in unlabeled proline for 2 min at 24 C and then chilled to 0 C, the chilling process taking about 5 min. C^{14} -proline was then added to the system, and a series of samples was taken. Figure 16 shows that the labeled proline entered

the trichloroacetic acid-soluble fraction of the cell, but almost no incorporation into the trichloroacetic acid precipitate occurred. It appears that the external C^{14} -proline exchanged with the C^{12} -proline that was previously adsorbed at 24 C and had remained in the pool during the chilling process. To show that exchange was occurring, a small amount of C^{12} -proline was added after equilibrium had been approached. The amount of C^{14} -proline in the pool then fell as a new exchange equilibrium was approached.

Table 3 shows the relative suppression of proline pool formation and exchange at 0 C for a given external concentration of proline.

If C^{12} -amino acid is not added before the sus-

pension is chilled, exchange is observed with the "native" pool that normally exists in the cell in the absence of supplement. In the case of proline, this very small pool has been estimated by means of exchange measurements at 0 C to be about 0.5 μ mole per g, dry weight. This result agrees

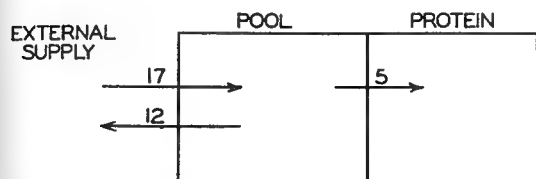


FIG. 14. Schematic diagram showing the exchange of pool valine during incorporation at a concentration of 0.3 μ g per ml. The numbers are flow rates in micromoles per gram of dry cells per 100 sec, calculated from the curve shown in Fig. 12.

with estimates made with growing cells at 25 C. In the case of valine, however, the native pool estimated by exchange at 0 C appears to be much larger than the native pool observed in growing cells at 25 C. This is in part due to the fact that valine can exchange with the leucine and isoleucine pools. In addition, however, excess valine appears to be synthesized by the cells during the cooling process. The excess valine was observed by chromatography of the pool of chilled cells. That pool formation did not occur at 0 C was indicated by a detailed study at that temperature of the quantity of C^{14} -valine appearing in the pool as a function of the concentration of external C^{14} -valine. The resulting data fitted exactly a curve calculated on the basis of exchange with a valine pool of 15 μ moles per g of dry cells. It is something of a mystery that the

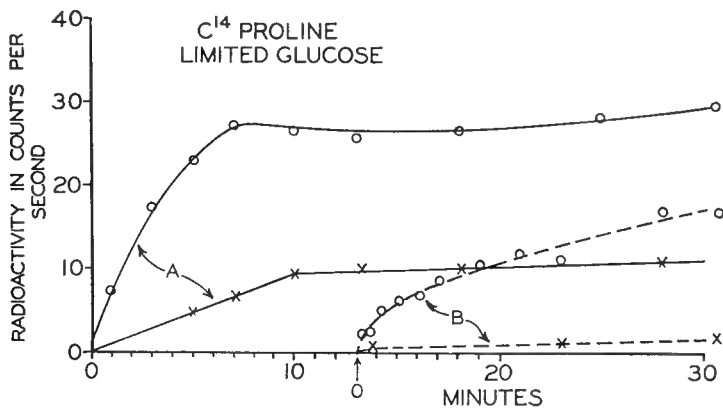


FIG. 15. Maintenance and exchange of pool proline in the absence of glucose. In both experiments, growing cells were suspended at time zero in medium containing 10 μ g per ml of glucose and 0.87 μ g per ml of C^{12} -proline. For curve A, a small quantity of C^{14} -proline was added at time zero. For curve B, an equal quantity of C^{14} -proline was added at 18 min. In each case the upper curve (O) represents the total C^{14} -proline taken up, and the lower curve (X), the C^{14} incorporated into protein. The difference is the C^{14} -proline in the pool.

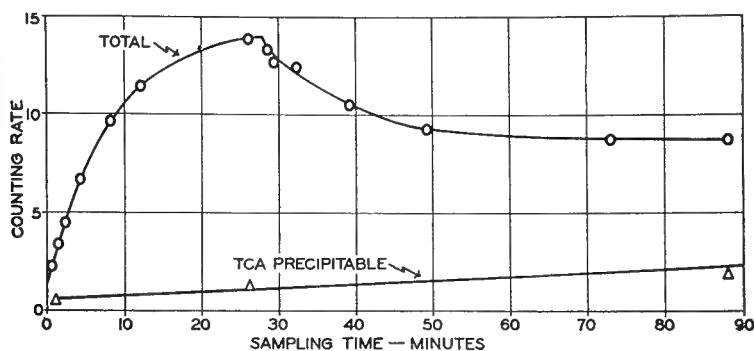


FIG. 16. Exchange between pool and exogenous proline at 0 C. At 28 min, C^{12} -proline was added. Circles represent total incorporation; triangles, incorporation into trichloroacetic acid (TCA) precipitate.

TABLE 3. *Approximate rates of formation and exchange^a*

Temperature	Rate of	
	Pool formation	Exchange
C		
0	0.0074	0.18
25	2.0	0.63

^a Results are expressed as millimicromoles of proline formed or exchanged per min per mg of wet cells at an external C¹⁴-proline concentration of 3.5×10^{-6} M. No glucose was present in the exchange experiments.

cells, during the cooling process, synthesize an excess of valine equal to several minutes of synthesis at the normal 25 C rate. Perhaps this point should be explored further. There is, however, no question that the suppression of amino acid pool formation at 0 C is a general phenomenon in *E. coli*.

In this context, it must be mentioned that the experiments of Cohen and Rickenberg (7) on the concentration of amino acids by *E. coli* were performed by chilling the suspension to halt the concentration process. These experiments have given a good picture of the concentration process and the interactions of a number of amino acids, but some details may have been blurred by exchange in the cold during centrifugation.

As mentioned above, we have observed that very large pools are unstable at 0 C. The saturation value of the valine pool quoted in (7) and (8) is about 20 μ moles per g of dry cells, measured by chilling and then centrifuging the cells. Using the filter technique, we have observed a saturation value of 60 μ moles per g of dry cells (10^{-4} M external valine concentration). This factor of 3 may, in part, be due to the different strains of cells used and to different temperatures. The osmotic strengths of the media were nearly identical, so that they should not have influenced the saturation pool size. It seems likely that a large part of the difference is, in fact, due to the different methods of measurement. It appears that the pool for a given amino acid is made up of more than one component (see Discussion below). The components have different specificity and exchange rates and presumably different stability toward chilling. As a result, the filter

and the chilling techniques may very well emphasize quite different aspects of pool behavior.

The specificity of the exchange process at 0 C also has been examined. Radioactive proline is not displaced from a pool in exchange equilibrium at this temperature by the simultaneous addition of 15 other nonradioactive amino acids, each at 100 times the proline concentration. Thus, the exchange process at 0 C is just as specific for proline as the pool formation process at higher temperatures. Exchange at higher temperatures must also be specific, since it plays an important part in the kinetics of pool formation. Excess leucine and isoleucine displace radioactive valine from a pool at 0 C in exchange equilibrium. The rate of displacement appears to be similar to that caused by the addition of excess C¹²-valine. Here again, the specificity of the exchange process appears to be similar to that of the pool-forming process.

Studies of the rate of exchange as a function of external concentration and pool size give surprising results, which yield insight into the exchange process and supply strong restrictions on models of the pool mechanism. For several reasons the rate measurements have been carried out at 0 C. It is convenient experimentally, since there is little incorporation into protein and the pools are stable for many hours. In addition it is possible to vary the pool size and external concentration independently. During pool formation at higher temperatures, the pool size will rise rapidly to the value dictated by the external concentration. However, at 0 C, the pool size will not show significant change for several hours, even when it is far from its normal value for the external concentration.

For these experiments, pools of a desired size were formed with unlabeled proline at 25 C. The suspension was then chilled to 0 C. The cells were centrifuged and resuspended in unsupplemented medium, and after approximately 1 hr at 0 C. C¹⁴-proline was added. Since the external quantity was small compared with the amount in the pool, a very efficient labeling of the pool by exchange was achieved. After a steady state (equal internal and external specific activities) was reached, the external concentration was brought up to a chosen value by adding C¹²-proline. The time course of exchange was then followed by measuring the loss of trichloroacetic acid-soluble radioactivity from the cells. The variation with

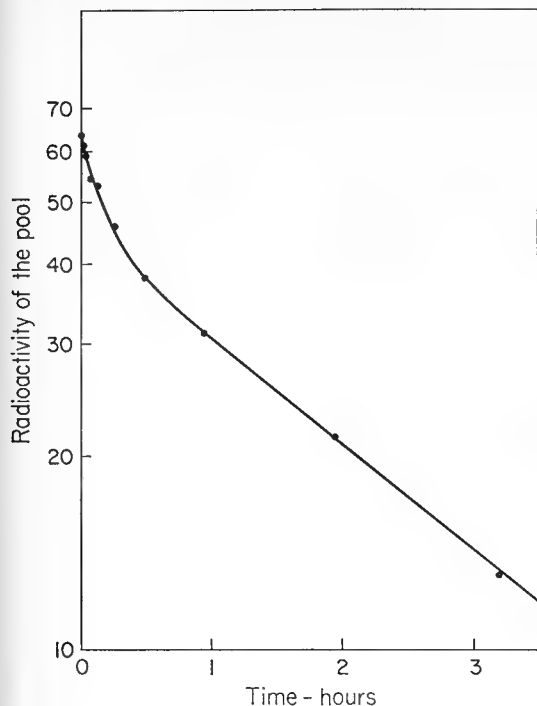


FIG. 17. Time course of exchange of the proline pool at 0 C. The log of the radioactivity of the pool is shown as a function of time after C^{12} -proline was added (10^{-4} M) to a suspension containing a C^{14} -proline pool of 2.9×10^{-6} mole per gram, wet weight, in equilibrium with external C^{14} -proline (1.9×10^{-6} M) at 0 C.

time of the logarithm of the radioactivity of the pool is shown in Fig. 17.

It is apparent that the time course of the exchange process does not follow a single exponential. It is possible, with good accuracy, to resolve this curve into two simple exponentials. When the results of such experiments over a wide range of concentration and pool size are examined, two components with widely different time constants can be resolved. A very definite conclusion can be drawn from these observations, owing to the simplicity of exchange processes. If a single, constant, homogeneous component exchanges with a constant quantity of amino acid in solution, the time course of the process must follow a simple exponential, i.e., display a single time constant. This statement holds regardless of the nature and multiplicity of the mechanisms mediating the exchange, as long as the quantity of amino acid associated with the intermediate steps is small. For example, it might be suggested that there were separate fast and

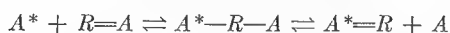
slow mechanisms which could act as intermediates in exchange between a single pool and the environment. However, the resulting time course would be a simple exponential with a time constant slightly faster than with the fast mechanism alone.

Thus it may be concluded that there are at least two separate components in the pool which are associated with the cell in different ways. The accuracy of these experiments is not sufficient to establish whether there are more than two components. The separate parts of the pool cannot exchange with each other at a rate faster than that shown by the slow component. The instability of the very large pools at 0 C suggests still another component in the pool.

The variation with total pool size of the exchange rates of both the fast and slow components is shown in Fig. 18. The number beside each point is the external concentration during exchange in micromoles per liter. It appears that the exchange rate is independent of the external concentration, except possibly at low concentrations.

The rapidly exchanging component of the pool is always smaller than the slowly exchanging one. It appears to saturate at less than $1.0 \mu\text{mole}$ per g of wet cells and is not easily observable when the total pool is greater than $10 \mu\text{moles}$ per g. The exchange rate of the large, slow component is roughly proportional to the total pool size. Unfortunately, the accuracy of the data is not quite sufficient to determine whether the exchange rate of each of the components is proportional to its own size, although this result is suggested by the evidence.

The exchange process may occur either through reactions that are an essential part of the overall mechanism of pool formation or through reactions that play no real part in that process. In connection with the latter case, it should be noted that a reaction of the type,



where $R \rightleftharpoons A$ is some complex containing A, would be observable in an exchange study but would not necessarily be observable in the process of pool formation, since the reaction causes no net change in the amount of the complex.

Any satisfactory model of the amino acid pool must certainly allow for the occurrence of exchange in the absence of an energy source and

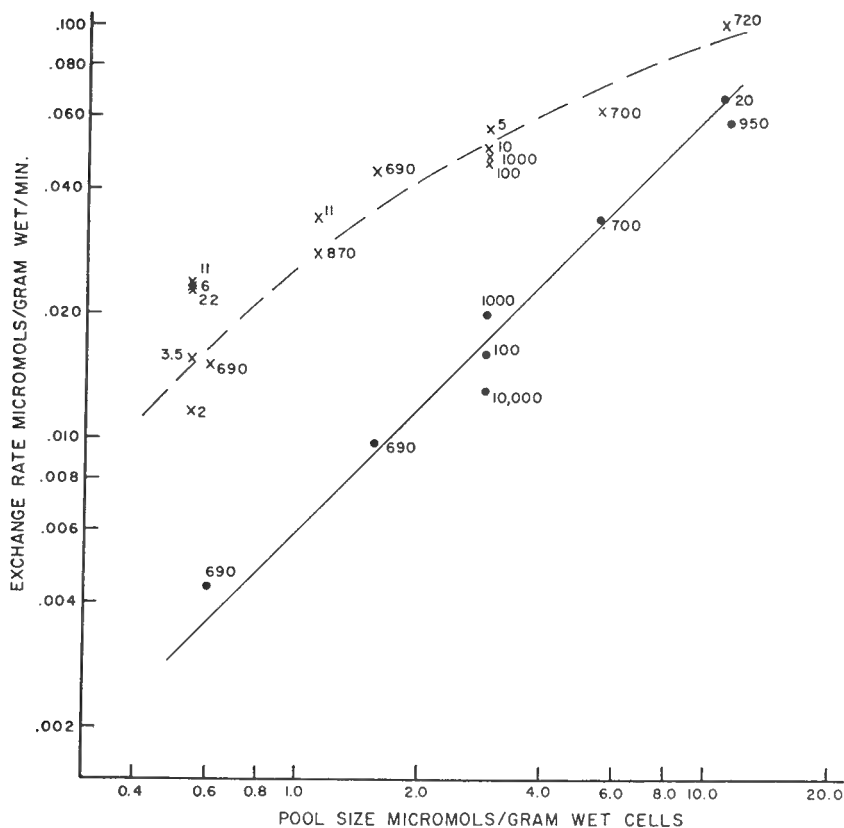


FIG. 18. Rate of exchange as a function of pool size (log-log plot). The points were obtained from experiments such as that shown in Fig. 17 by fitting the time course of exchange to curves derived from the sum of two exponential decays. The numbers beside each point are the external concentrations during exchange, in micromoles per liter. The straight line shown would result if the exchange rate were proportional to pool size.

for the strikingly different temperature dependence of the exchange process and the process of pool formation. A model of the pool mechanism should also have features which limit the rate of exchange and should suggest how the rate of exchange can be independent of the external concentration but proportional to the pool size. Finally, a sophisticated model should indicate how the different components of the pool differ in their association with the cell.

I. Variation of the Pool Size and Formation Rate with the External Concentration

Measurements of the pool size and the rate of formation of the pool as a function of external concentration are valuable since they may be compared quantitatively with calculations based on models. It would be in keeping with the traditions of enzyme chemistry if these obvious features of the pool could be pigeonholed by simply

determining the Michaelis constants for the interaction of amino acids with the cell. However, pool formation is an energy-coupled process of a whole living cell, and it is not surprising that such simplicity is lacking.

A survey of a large number of exploratory experiments done for many other purposes indicated that the pool size did not rise quite in proportion to the external concentration at low concentrations (far below saturation of the pool). It also appeared that the rate of formation of the pool reached a maximum at an external concentration much lower than that at which the pool size reached its maximum value. There was, however, a large amount of scatter in the measurements of both the pool size and rate of formation. In order to avoid sources of variation, measurements of the kinetics of proline pool formation were carried out simultaneously at seven

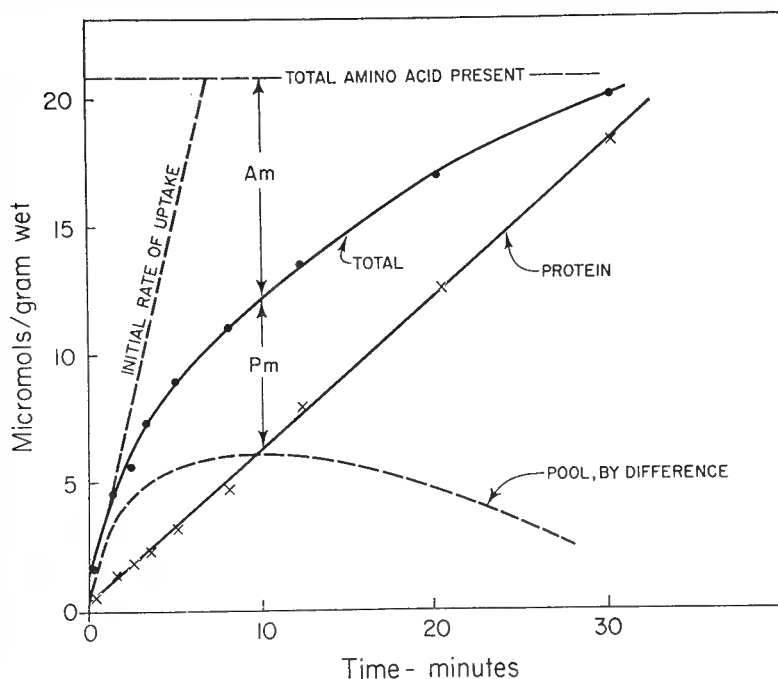


FIG. 19. Kinetics of proline pool formation at 25 C. Exponentially growing cells (0.48 mg, wet weight, per ml) were supplied C^{14} -proline at 1.0×10^{-5} M. The initial total rate of uptake may be calculated from the dashed line. The maximum pool size and the external concentration at the time it was achieved are determined from the values, P_m and A_m , shown.

different concentrations with samples from the same suspension of growing cells.

Figure 19 shows the kinetics of pool formation at an intermediate concentration (10^{-5} M). The lower curve shows the variation of the pool with time, achieving a maximum at about 10 min and slowly falling at later times. When the pool reaches its maximum value¹ (shown as P_m) its rate of change is zero, and therefore it has the steady value corresponding to the external concentration at that moment (shown as A_m).

The variation of the pool size with the external concentration determined from the seven simultaneous measurements is shown in Fig. 20. The dashed curve represents a classical adsorption isotherm (saturation value, $S = 70$ μ moles per g of wet cells; $K_s = 4 \times 10^{-5}$ M) fitted to the points at higher concentrations. At lower concentrations, the measured values of the pool are 3 times larger than those given by this isotherm. It is possible to fit the experimental curve with the sum of two isotherms. The results of a more

thorough analysis are presented in the mathematical appendix. Thus the variation of pool size with concentration is consistent with the presence of more than one component in the pool. It does not, by itself, demonstrate this, since there is no independent evidence that individual components would follow the classical adsorption isotherm.

Two methods have been used to measure the initial rate of uptake of amino acid by the cells in this set of experiments. With the first method, a direct estimate of the total rate of incorporation was made from the early time points as shown by the dashed line in Fig. 19. By the second method, the difference between the maximum value of the pool and the pool at any time was plotted on logarithmic paper. A straight line results. In other words, the pool rises with time approximately as $P = P_m (1 - e^{-t/T})$. From the empirical time constant, T , and P_m , the initial rate of increase of the pool can be calculated. The initial total rate of uptake is determined by adding this figure to the rate of incorporation into protein. The two methods are in close agreement.

¹ See the mathematical appendix (Part IV) for a more precise method of evaluating the pool size at low concentrations.

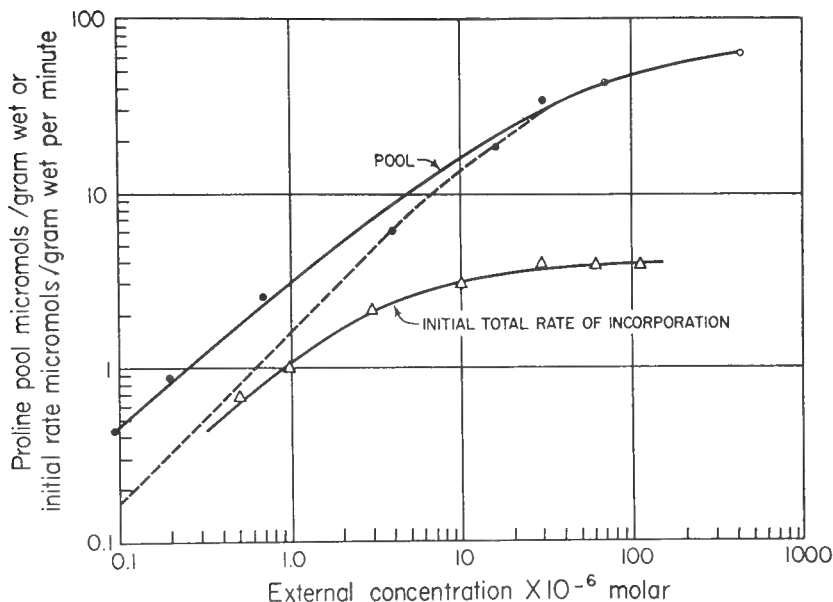


FIG. 20. Log-log plot of the proline pool and initial rate of incorporation as a function of proline concentration at 25 C. The upper points (●) represent the results of a set of simultaneous measurements of pool size in one experiment as described in the text. The open circle (○) represents the results of a number of measurements of the saturation value of the proline pool. The dashed curve is an adsorption isotherm: $P = \pi A / (A + k_s)$, with $\pi = 70 \mu\text{moles per g of wet cells}$ and $k_s = 4 \times 10^{-5} \text{ M}$. The lower set of points (Δ) is the result of the measurement of the total initial rate of incorporation of proline.

As the curves in Fig. 20 show, the variation of the initial rate of incorporation with concentration is much less than the variation of the pool size. Between 10^{-6} and 10^{-4} M , the pool increases by a factor of 15, while the initial rate of incorporation increases by only a factor of 4. The measurements of the initial rate of incorporation fit an adsorption isotherm (shown by solid line) with $K_s = 2.5 \times 10^{-6} \text{ M}$. The measurements of the pool size, at the larger concentrations, fit an adsorption isotherm (shown by the dashed line) with $K_s = 4 \times 10^{-5} \text{ M}$. Thus the pool itself saturates at a concentration more than 10 times the concentration at which the rate of pool formation saturates.

This pair of observations, by itself, is sufficient to eliminate the simpler models of the concentrating mechanism. As a result, it is worthwhile to consider whether any systematic errors are present which might weaken such an argument. With regard to the measurements of rate of incorporation, the process is relatively slow at the higher concentrations and there appears to be little source of error. However, at the lower concentrations, the rate falls rapidly during the first minute, and the measurements probably indicate rates that are somewhat slower than

the actual rate at zero time. Again, for the pool size, the measurements at high concentrations are better, since the external concentration is changing slowly and one can be more certain of the external concentration at the time the pool has reached its maximum value. Since the very large pools are less stable, there is a chance that some part may be lost during the filtering process, but there is no evidence that this is so. At the lowest concentration used, the pre-existing native pool (measured to be $0.2 \mu\text{mole per g of wet cells}$ in this experiment) is significant compared with the labeled pool formed. In addition, internal synthesis continues (in the experiments at the lower concentrations) during the time required to form the pool. Thus the labeled amino acid is somewhat diluted in the pool. The pool size at low concentrations is therefore somewhat larger than that estimated by the method used for Fig. 20. When correction is made for these effects (see Fig. 31), the deviation of the measured curve from a single adsorption isotherm is increased. Further, since exchange occurs between the pre-existing pool and added amino acid, the measured total rate of incorporation is somewhat greater than that due simply to uptake of the amino acid. The lowest

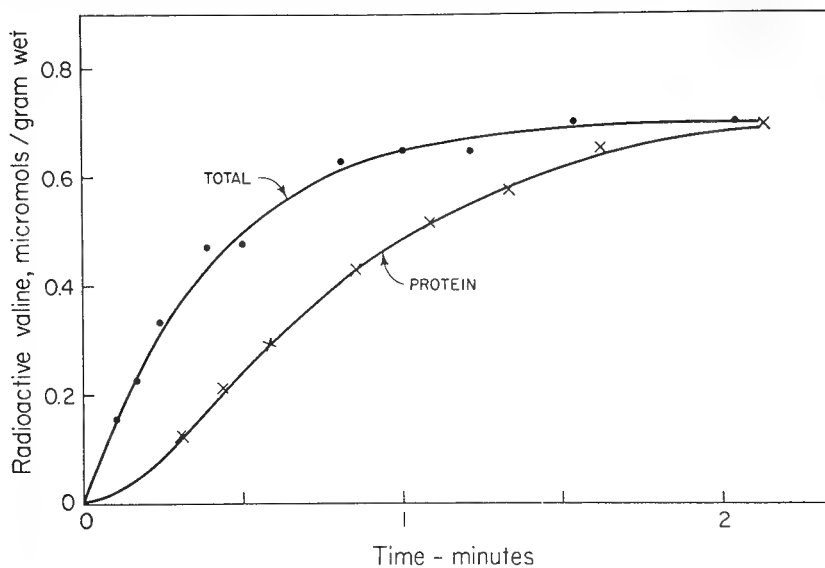


FIG. 21. Kinetics of valine incorporation at a very low concentration. Exponentially growing cells (0.08 mg, wet weight, per ml) at 25° C were supplied C^{14} -valine at 5.6×10^{-8} M. The initial total rate of uptake is one-tenth of the maximum rate allowed by diffusion. The internal concentration, averaged over the whole cell volume, rises to 18,000 times that present externally.

point (Δ) could not be in error by more than 50%, and this would not have a large effect on the apparent K_s .

It appears that the large difference between the concentrations at which the pool size and rate of formation saturate is definitely established and must be taken into account in building models of the process of pool formation.

To complete the comparison of the rate of formation and pool size, and for reasons of general interest, an example of the uptake of valine at an extremely low concentration is shown in Fig. 21. Valine at 5.6×10^{-8} M was supplied to an exponentially growing culture at 0.08 mg of wet cells per ml. The low cell density was used to reduce the rate of incorporation and the quantity of pre-existing valine in the suspension. The maximum rate of incorporation of C^{14} -valine into protein shows that at 30 sec, when the pool was maximal, the initial specific radioactivity had been diluted by about 30%. Thus there can be no major error in the original external concentration caused by valine pre-existing in the medium. The general shape of the curve is precisely that to be expected if a small unlabeled pool were initially present and valine continued to be synthesized by the cells. There is an initial lag in protein incorporation as the specific radioactivity of the pool rises, and then the rate at

which C^{14} -valine enters the protein falls as the labeled pool is diluted by internal synthesis. Valine was chosen for this experiment, even though some confusion results from its conversion to leucine, because it was known to be incorporated extremely rapidly at low concentrations.

The initial rate of incorporation in this experiment, determined as above, was 2 μ moles per g of wet cells per min. This impressively high rate of uptake represents the removal of all of the valine in 600 cell volumes of medium in 1 sec by each cell. Calculation shows that the equilibrium rate of diffusion into a sphere of volume equal to one cell (continuously maintained at zero internal concentration) would result in the uptake of all of the valine in 5,400 cell volumes of medium per sec. Thus the flow of valine into the cell occurs at a rate about one-tenth of the maximum rate allowed by diffusion.

A few measurements of the incorporation of valine have been made at higher concentrations. Table 4 gives the results of measurements at the extremes of the concentration range that has been examined and of a pair in the median region. Values in parentheses are less certain. The pool size changes by a factor of 14, while the initial rate of incorporation changes by

TABLE 4. Valine pool and rate of formation

Valine concentration	Pool size	Initial rate of incorporation	Concentration ratio ^a
$\mu\text{moles/liter}$	$\mu\text{moles/g wet}$	$\mu\text{moles/g} \times \text{min}$	
70.0	15 ^b		210
10.0	(10)	5.0	
2.9	6.5		2,200
1.3		3.0	
0.056	(0.7)	2.0	
0.016	0.3		18,000

^a Obtained by: (pool per ml of cells)/(valine per ml of medium).

^b Maximum pool.

only a factor of 2.5 over the concentration range from 0.056 to 10 μmoles per liter. In addition, the pool size measured at 0.016 μmole per liter is 5 times the value predicted by an adsorption isotherm fitted to the points at higher concentrations.

It is clear that for valine, just as has been shown previously for proline, the pool size as a function of external concentration does not follow a classical adsorption isotherm, and the concentration dependence of the initial rate of incorporation is very different from that of the pool size.

J. Loss from the Pool after Dilution of the External Amino Acid

A few exploratory measurements have been made of the rate of loss from the pool when the external amino acid concentration is suddenly reduced. These experiments show that the rate of loss from a given pool at 25 C is very much slower than its rate of formation. The existence of a rate of loss much slower than the rate of formation sets a stringent requirement for models of the process.

The rate of loss to the external environment is so slow that it cannot be measured when the pool is being utilized for protein synthesis. In the three experiments described below, protein synthesis has been inhibited in three ways: by reducing the temperature to 0 C, by removing glucose at 25 C, and by removing required supplements from a deficient mutant.

The experiment at 0 C is not easily interpretable, since all of the processes of pool formation and maintenance are strongly modified by the low temperature. Nevertheless, this experiment is worth discussing briefly. Figure 22 shows

the results, and the legend describes the method used. A loss rate of 2% per min is observed in C and D. This rate is faster than that observed at 25 C (see below) from a somewhat larger pool. Curiously, it is also faster than the initial pool formation rate at 0 C (0.3% per min) measured in the experiment of Fig. 5. Since the pool sizes in these two cases are the same within a factor of about 2, this result is very difficult to explain. Until further experiments are carried out, only the qualitative result that the loss rate may be very different from the formation rate at 0 C can be used in arguments concerning the mechanism of pool formation.

In order to measure the rate of loss in the absence of glucose at 25 C, growing cells were supplied C¹²-proline at 10⁻⁴ M for 10 min, centrifuged, and resuspended in the absence of both glucose and proline. Five minutes later, C¹⁴-proline was added, and the pool rapidly became labeled by exchange (half-time to equilibrium, about 2 min). No incorporation into protein could be measured. Ten minutes later, when exchange equilibrium was established, samples were collected on collodion filters and washed continuously on the filter with unsupplemented medium for periods up to 10 min. There was a very fast initial loss of about 10% of the pool and no further measurable loss.

The measurement² at 25 C in the presence of glucose was carried out with the mutant 15 T-A-U⁻ in the absence of its three required supplements (thymine, arginine, and uracil). Exponentially growing cells at 25 C (supplemented with arginine, thymine, and uracil) were harvested on a collodion membrane filter and washed with unsupplemented medium. The cells were immediately resuspended (0.5 mg of wet cells per ml) in the presence of glucose but without arginine, thymine, or uracil. It was felt desirable to complete the experiment during the 50 min (at 25 C) before thymineless death begins in this strain. Therefore, the C¹⁴-proline was added about 10 min after the cells were resuspended in the absence of thymine. The

² While this section was being written, it became apparent that the point was important enough to require an additional experiment. The authors would like to express their appreciation to O. Maaløe at the Microbiological Institute of the University of Copenhagen for the use of the facilities at his laboratory and for the purchase of C¹⁴-proline.

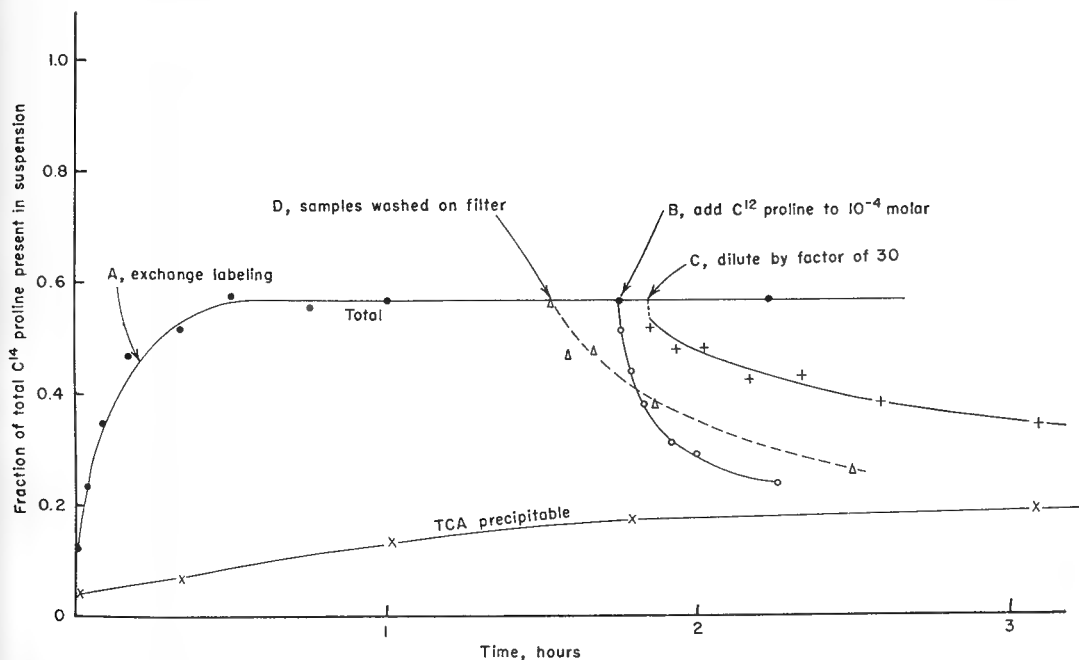


FIG. 22. Study of rate of exchange and loss of C^{14} -proline pool in *E. coli* at 0 C. Cell concentration, 1.0 mg of wet cells per ml. The suspension was incubated at 25 C for 5 min after addition of 10^{-5} M C^{12} -proline and quickly chilled to 0 C. The cells then were centrifuged and resuspended in unsupplemented medium at 0 C. After 1 hr, C^{14} -proline was added in order to label the preformed pool by exchange (A). To an aliquot of the suspension (B) carrier proline was added to a concentration of 10^{-4} M. Another aliquot (C) was diluted by a factor of 30 with unsupplemented medium. Finally (D), samples were filtered and washed continuously on the filter for the times indicated, with unsupplemented medium at 0 C.

upper curve (●) in Fig. 23 shows the kinetics of pool formation.

Eleven minutes after the C^{14} -proline was added, 20 ml of the culture were injected with a hypodermic syringe into 280 ml of medium supplemented only with glucose. The lower curve (+) shows that the total radioactivity in the cells hardly changes during the succeeding half hour. There is no measurable loss from the pool. The maximum possible loss rate consistent with the scatter in the measurements would be about $0.02 \mu\text{mole per g of wet cells per min}$ or 1% of the initial rate of formation.

These two experiments demonstrate that the rate of loss from the pool is very slow at 25 C when the external amino acid concentration is greatly reduced, whether or not glucose is present. Clearly, then, the outflow of amino acid from the pool must also have been small before the amino acid concentration was reduced.

This result implies that when a steady-state pool has been achieved, the circulating flow of amino acid between the pool and the medium

must be small. In other words, the rate of entry of amino acid into the pool (which was initially large) must be strongly suppressed as the steady-state pool size is approached. In contrast, in a bimolecular chemical reaction, $A + B \rightleftharpoons AB$, the rate of formation of AB is constant as long as the concentrations of A and B remain constant. As equilibrium is approached, the rate of dissociation of AB rises until it equals the rate of formation.

In pool formation we are therefore dealing with an unusual type of process. A close analogy would perhaps be a centrifugal pump filling a reservoir (without significant leak) up to the limiting pressure that the pump can deliver. Such a pump might very well give a slow leak rate if the supply pressure were suddenly reduced. Most simple models for the concentrating process do not have this feature. The fairly complex carrier model, described later, does behave in just this way.

We should be reminded that exchange experiments, on the contrary, indicate a large circula-

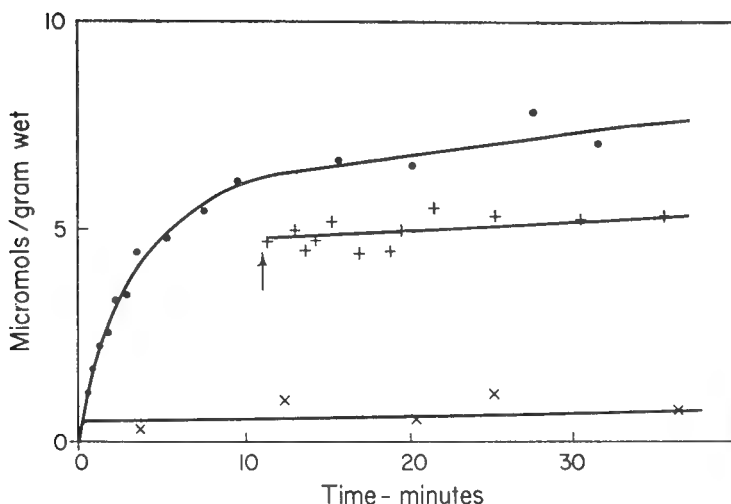


FIG. 23. Stability of the proline pool after reduction of the external concentration. *E. coli* strain 15 T⁻A⁻U⁻ suspended at 0.5 mg, wet weight, per ml at 25 C in the absence of thymine, arginine, and uracil. Glucose was present at 2 mg per ml. At time zero C¹⁴-proline was added (●) to a concentration of 10^{-5} M (20 μ moles per g of wet cells). At 11 min (shown by arrow), part of the culture (+) was diluted by a factor of 15 and correspondingly large samples were taken. The concentration of C¹⁴-proline after dilution was 5×10^{-7} M. The incorporation into protein is shown by the lower set of points (X). The initial rate of uptake was 1.5 μ moles per g of wet cells per min. The rate of loss from the pool is not measurable, but certainly less than 1% of the rate of uptake.

tion of amino acid when a steady pool exists at 25 C. Clearly this *must* occur by a mechanism independent of the *active* concentration process. The independence of the exchange process has been directly demonstrated at 0 C (Table 3), where the exchange rate is 25 times the formation rate. A large excess of exchange rate over formation rate is also observed at 25 C in the absence of glucose.

K. Osmotic Properties of the Pool

During the course of studies of the extraction of the amino acid pool by various agents, it was observed that distilled water completely removes the amino acid pool (5). However, when the bacteria were returned to their normal growth medium after treatment with distilled water, they almost immediately (within 2 min) resumed their normal rate of protein synthesis and rate of uptake of amino acids into the pool. These observations led to an experimental study of the osmotic properties of the pool.

Pool removal as function of osmotic strength of washing medium. A rapidly growing culture of *E. coli* cells was supplied C¹⁴-proline and, after the pool size had reached an approximately steady value, a series of samples was withdrawn and filtered on collodion membrane filters. The thin

layer of cells collected on the filter (1 to 2 mg of wet cells) was then washed by drawing 2 ml of a given solution through the filter in 5 to 10 sec. Assays of the radioactivity of unwashed cells and of those washed with unsupplemented growth medium showed no significant difference, indicating that the quantities of amino acid in the pool were very large compared with the holdup of labeled medium on the filter and cell pad, and that little loss from the pool occurred. The fraction of the pool removed was determined by comparing the radioactivity remaining after various washes with that remaining after a trichloroacetic acid wash. Figure 24 shows the results of such experiments. The fraction of the pool remaining is plotted against the "osmotic strength," that is, the total molar concentration of solute species in the washing solution, taking account of ionization. A relatively mild osmotic shock removes amino acid from the pool. For example, a 30% reduction in the osmotic strength will remove 40% of the pool. Washing with various concentrations of glucose removed about the same fraction as that removed with salt of the same osmotic strength. When washes are performed with 0.37 osmolar solutions of glucose, sucrose, glycine, or NaCl, the pool is unaffected. Washing with glucose solutions of higher osmolar-

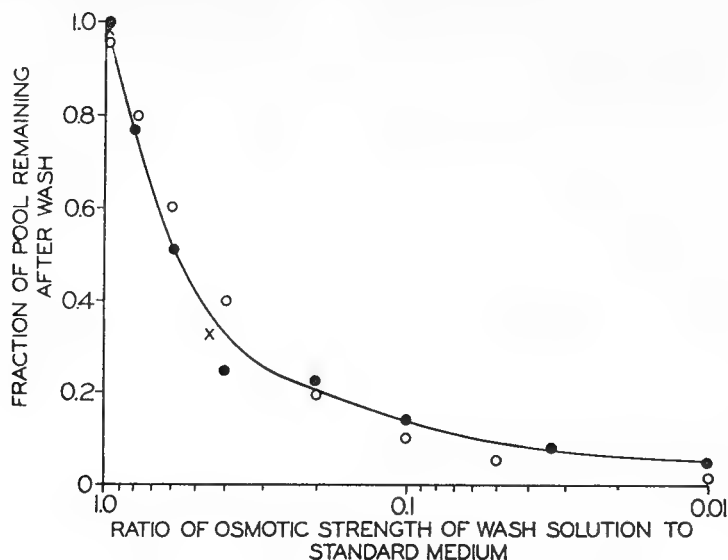


FIG. 24. Removal of pool by washing cells with solutions of low osmotic strength. Growing cells were supplied C^{14} -proline and allowed to form a pool, and then samples were collected on a membrane filter. Various wash solutions were then passed through the cell pad on the filter. The points marked (●) were washed with various dilutions of the standard growth medium, the points marked (○) with NaCl solutions, and the points marked (×) with glucose solutions.

ity than 0.37 has little effect on the pool. Similar results have been obtained whether the tests were carried out with large proline pools (50 μ moles per g of dry cells), small proline or valine pools (1 to 10 μ moles per g of dry cells), or on the complex pool internally synthesized from C^{14} -glucose. These results show that it is the osmotic and not the ionic strength of the washing medium that determines the fraction of the pool that is removed.

Maximum pool size as function of osmotic strength of medium. When pool formation was carried out in solutions of various osmotic strengths, it was found for small pools (< 5 μ moles per g of dry cells for a given amino acid) that the pool size was independent of the osmotic strength of the medium. However, the maximum pool size (high external amino acid concentration) is strongly dependent upon the osmotic strength of the medium. Figure 25 shows the results of a series of experiments in which the saturation pool size was measured in growth media of various osmotic strengths. The maximum pool is nearly proportional to the osmotic strength of the medium. This striking observation stands at the moment entirely without explanation.

Recovery from osmotic shock. In order to assess the type of damage caused by a sudden decrease

in osmotic strength (osmotic shock), the ability of the cells to form pools and synthesize protein was measured immediately after a water wash.

A centrifuged pellet of cells from an exponentially growing culture was suspended in a small volume of water and quickly (<10 sec) added to a beaker containing the usual growth medium supplemented with C^{14} -proline. Pool formation started after a delay of less than 1 min, and protein incorporation achieved its normal value after about 1½ min. Thus, complete removal of pool compounds by the violent osmotic shock is due to a very transient change in the cell. Synthesis of any significant fraction of the cell protoplasm is not necessary in order to repair the damage.

Figure 26 shows the results of another type of experiment illustrating this same effect. While incorporation of C^{14} -proline was in process, the suspension was diluted with 2 volumes of water at the same concentration of C^{14} -proline. At the instant of dilution, the pool size dropped sharply and after 1 min started back up toward its original value.

Descriptive model of process of pool removal by osmotic shock. Speculation regarding these observations has led us to the following tentative model of the phenomenon of osmotic shock.

It is assumed that the cell contains osmotically

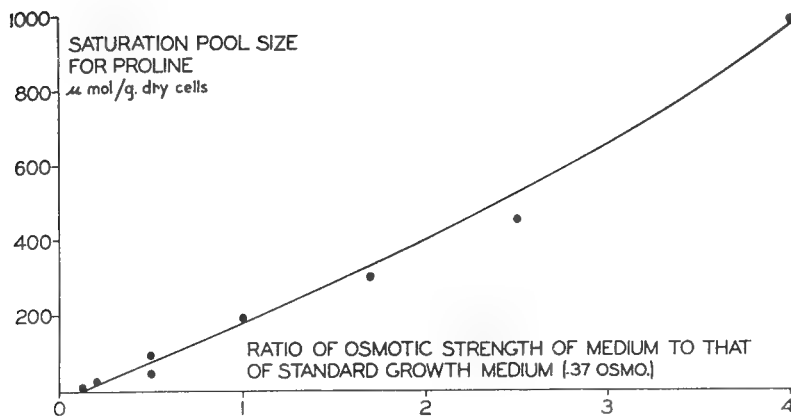


FIG. 25. Saturation pool size as a function of the osmotic strength of the medium. The osmotic strengths shown were obtained by adding glucose to a 1:10 dilution of the usual growth medium. Growth is inhibited at the higher osmotic strengths.

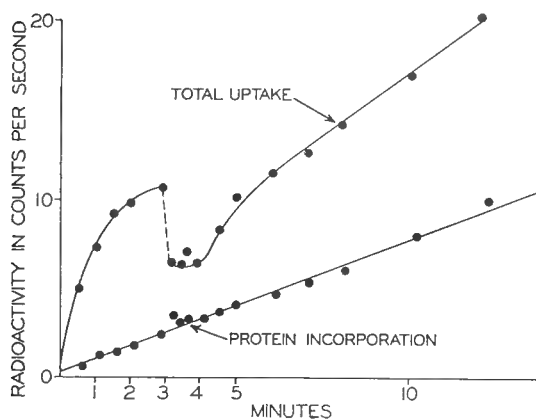


FIG. 26. Recovery of pool, after removal by osmotic shock. At time zero, $0.3 \mu\text{g}$ per ml of C^{14} -proline was added to 0.08 mg , dry weight, per ml of growing cells. At 3 min, 2 volumes of water containing $0.3 \mu\text{g}$ per ml of C^{14} -proline were added.

sensitive structures which are initially in osmotic equilibrium with the external medium. When the external osmotic strength is suddenly reduced, there is a flow of water into the osmotically sensitive structures along with a slow loss of solute from the cell. The consequent stretching of the structures due to the internal pressure increases the permeability to the solute, allowing a faster rate of loss of solute molecules. These two processes, together, lead finally to a new osmotic equilibrium. During this process, the cell passes through a transient state in which the structures are distended. The loss of pool is associated with this transient distention of the cell structures. The evidence concerning the osmotic sensitivity

of the pool does not make it possible to decide whether the structures involved are the cell wall and membrane, structures within the cell, or a combination of these.

Pool removal versus rate of shock. Since competing rates of flow of water and solute are involved, this model suggested that a slow change in the osmotic strength might be less effective than a rapid shock. In an experiment with a large proline pool, a reduction in osmotic strength of a factor of 3 was made in four steps of equal concentration ratio. As a result, 70% of the pool was removed. If the same final osmotic strength was achieved in a single step, 90% was removed. Further, when the same final osmotic strength was achieved through a slow concentration change graded continuously over several minutes, only 50% of the pool was removed.

Uptake during shock. Another implication of this description of the process of osmotic shock is that molecules which do not ordinarily enter the osmotically sensitive structures might be able to diffuse in, during the transient period when the permeability is increased. We call this process "trick or treat," since it was suggested just after Halloween and brought to mind the children's trick of throwing in orange peels while the door is open.

This transient permeability was tested experimentally by giving a thick suspension of cells at 0°C a sudden osmotic shock in the presence of radioactive SO_4^{2-} or PO_4^{3-} . The suspension was then diluted without osmotic shock to remove diffusible label and filtered. A small amount of SO_4^{2-} or PO_4^{3-} was taken up corresponding to

about 5% of the cell volume at the external concentration of $\text{SO}_4^{=}$ or $\text{PO}_4^{=}$. Various controls showed that this uptake was indeed due to the sudden downward change in osmotic strength. Upward osmotic shocks neither remove the pool nor cause the "trick or treat" phenomenon.

This phenomenon may shed some light on earlier observations of the paradoxical conditions required for efficient production of mutations with Mn^{++} (9, 13). Effective production of mutations occurred when cells suspended in saline were transferred to a low osmotic strength medium containing Mn^{++} .

Dependence of osmotic shock on nature of solute. Studies have been made of the effectiveness of various solutes for producing or preventing osmotic shock. The results are summarized in Table 5. Since, in general, upward osmotic shock has no effect on the pool, the figures in column A simply indicate any chemical or destructive effects on the cells. Thus the compounds such as butanol remove most of the pool. The figures in column B indicate the effectiveness of the solute as an osmotic protector for short periods of time. It will be seen that, in general, higher molecular weight compounds are most effective, although the zwitterion glycine (mol wt, 75) is an effective protector, while glycerol (mol wt, 92) is not. It is presumed that solutes which do not act as protectors are able to enter the osmotically sensitive structures rapidly. The resulting excess water activity outside causes water to flow in until the distention allows internal osmotic constituents to leak out, re-establishing equilibrium.

Column C shows the effect of a sudden doubling of the osmotic strength followed rapidly by a return to the usual medium. Compounds such as acetone, which enter the cell rapidly without causing damage (as shown by the figures in columns A and B), do not cause significant removal by downward osmotic shock. On the other hand, compounds such as urea and glycerol, while still not effective protectors, are capable of removing the pool by osmotic shock.

These results indicate that, by using the removal of the pool as an index of the osmotic state within the structures, studies might be carried out on the rates of entry of a variety of small molecules.

Dependence on nature of pool. Different types of pools appear to have different sensitivity to

TABLE 5. *Osmotic effects of various solutes on E. coli proline pool*

Solutes	Percentage of pool removed		
	A, upshock ^a	B, protection, ^b	C, upshock and downshock ^c
Butanol.....	77	100	77
Diethylene glycol.....	30	70	95
Ethyl acetoacetate.....	50	90	50
Methanol.....	20	100	20
Ethanol.....	20	100	20
Propanol.....	20	100	20
Acetone.....	10	100	15
Propionamide.....	10	100	20
Succinimide.....	20	100	20
Acetamide.....	20	100	50
Dioxan.....	5	95	40
Glycerol.....	5	99	70
Urea.....	5	97	63
NaCl.....	0	38	55
Na acetate.....	3	60	50
Diethylamine-HCl.....	6	35	60
Tris ^d -HCl.....	8	35	55
Glycine.....	1	16	47
Alanine.....	3	12	50
Valine.....	5	12	60
Proline.....	5	30	60
Glucosamine-HCl.....	0	5	50
Xylose.....	0	10	52
Glucose.....	0	10	52
Galactose.....	0	10	45
Sucrose.....	0	5	70

^a Washed on filter with solute at 0.37 osmolar concentration in growth medium.

^b Washed on filter with solute at 0.37 osmolar concentration in H_2O .

^c As in A, followed by wash with growth medium.

^d Tris(hydroxymethyl)aminomethane.

osmotic shock. Both amino acids and phosphorus compounds (measured after labeling with P^{32}O_4) are completely removed by a quick water wash at room temperature. However, at 0 C, the amino acid pools are still completely removed, while only half of the phosphorus compounds are removed. Osmotic shocks (at room temperature) that remove half of the amino acid pool will remove considerably less of the phosphorus compounds. It also appears that when very large amino acid pools (formed at high osmotic strength from a casein hydrolyzate supplement) are partially removed by osmotic shock, the amino

acid distribution in the pool is considerably altered. Thus, the amino acids in the pool show different sensitivities to removal by osmotic shock. These observations indicate that various pool materials are organized in different ways within the cell, and perhaps are associated with different substructures.

Removal of other soluble components by shock. Some studies have been made, by means of freezing point determinations, of the release of the total osmotically active material of the cell by osmotic shock. The results are broadly similar to those of studies of amino acid pools. Water washes remove the total osmotically active constituents almost completely. Boiling of the cells, after water washing, releases only traces of additional material effective in depressing the freezing point of water. When the osmotic shock is performed in small steps, the release of the total osmotically active material is quite similar to the release of amino acid pools, though perhaps a somewhat greater percentage is released for the same shock. The total quantity released from the cell indicates that if this material is osmotically active when present in the cell, the osmotic pressure within the cell is slightly greater than that of the medium and is dependent on the osmotic strength of the medium. If this material were concentrated in regions smaller than the whole cell, the osmotic pressure would be proportionately higher.

In this section, we have summarized our observations of the osmotic properties of pools in *E. coli*. While we have indicated how we picture the process responsible for the transient effects observed, the model cannot be considered unique, nor has it been developed sufficiently to test its ability to explain the results quantitatively. However, any complete model of the processes of pool formation and maintenance must be consistent with the osmotic behavior described.

L. Pools Formed in the Absence of Supplements

The "native" pool existing in a suspension of cells growing in an unsupplemented medium is the result of a balance of rates of synthesis and utilization of the amino acids. It is known that a moderate expansion of the pool by supplements will almost completely suppress the synthesis of certain amino acids (14). Thus, the native pools are probably an important link in the system

that controls the rate of synthesis of low molecular weight compounds.

Estimates of the size of the native pools have been made in a few cases by adding labeled amino acid at a very low concentration. Such small quantities of amino acid are rapidly taken up into the cell, and the rate at which the label enters the protein, compared with the known rate of incorporation of the amino acid, gives a measure of the native amino acid dilution of the supplement and, by calculation, the pool size. In the cases of proline, valine, leucine, isoleucine, methionine, and tyrosine at 25 C, the label enters the protein at a rate that would lead to its exhaustion in a time between 30 and 60 sec. Thus the quantities range between 0.5 and 2 μ moles per g of dry cells for these amino acids. The label in glutamic acid, however, is exhausted only after about 10 min under these conditions, and therefore its native pool size is about 50 μ moles per g of dry cells. It does not seem worth while to quote the precise results of these measurements, since they are quite variable, and no detailed studies of the causes of variation have been carried out. Native pools for valine have been observed as large as 15 μ moles per g of dry cells, although they are normally one-tenth of this size.

These native pools normally exist in equilibrium with a quantity of amino acid released to the medium. Measurements of the internal concentration relative to that present externally in unsupplemented cultures have not been carried out for growing cells. However, the fate of a very small quantity of glucose (described in the section on energy requirement) shows that the concentration ratios are very high for some amino acids in the absence of glucose. The figure for valine is 28,000:1, in the absence of glucose. It is greater than 18,000:1 in the presence of glucose, as shown by the experiment of Fig. 21.

M. Miscellaneous Observations Related to the Pool

In the studies of the pool formation process, a number of incidental observations have been made which do not at the moment lead to any definite conclusions. Nevertheless, it seems worthwhile to put them on the record in this section.

Since chloramphenicol is thought to suppress specifically protein synthesis, it might seem to be a useful tool for the study of pool formation. It would be expected to increase the pool somewhat

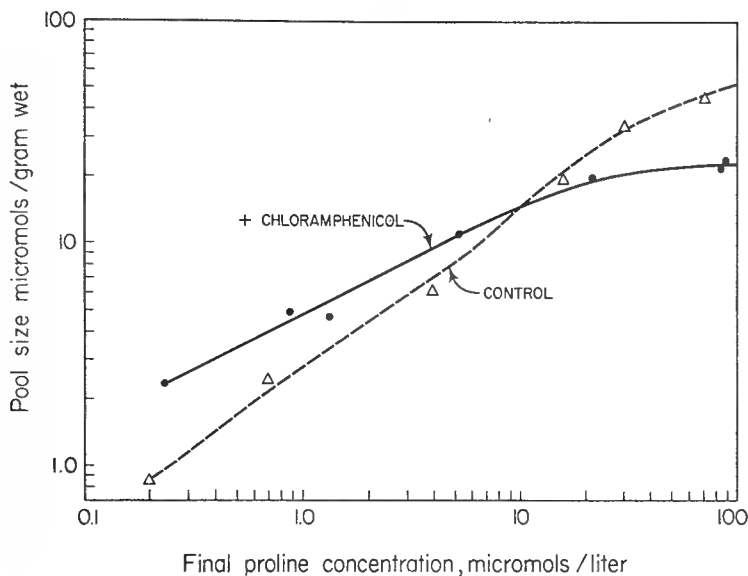


FIG. 27. Effect of chloramphenicol on the proline pool. Chloramphenicol, 20 μ g per ml, was added to a growing culture of cells at 25 C. Five minutes later the appropriate concentration of C^{14} -proline was added. The kinetics of pool formation were followed, and the pool size and external concentration were estimated after the pool had leveled off at its maximum value. The results are shown by the solid circles and solid line. The open triangles and dashed line show the pool size for growing cells from Fig. 20.

since the drain from the pool into protein would not be present. The observations, however, show that it interferes in some way with the formation of large pools. Figure 27 shows the results of a study of the proline pool size as a function of concentration in the presence of 20 μ g per ml of chloramphenicol. For comparison the pool size in growing cells is shown (Δ) by the dashed line. It appears that the maximum pool size is reduced by a factor of more than 2 by chloramphenicol. An increase in the pool size at low concentrations is observed as expected from the removal of the drain from the pool into protein.

In magnesium-deficient media, the ability to form amino acid pools in *E. coli* was markedly reduced, as was the rate of incorporation into the trichloroacetic acid-precipitable fraction. Reductions of as much as a factor of 5 in pool size have been observed, but the results are quite variable, presumably as a result of uncontrolled traces of Mg present in the "Mg-deficient" media.

When *E. coli* cells are exposed for a few minutes to a hydrostatic pressure of 20,000 psi, a large part of the amino acid pool is released to the medium. Even though growth of the cells is inhibited for an hour or so after the pressure is

removed, most of the released pool is quickly reincorporated. The sensitivity of the pool to hydrostatic pressure presumably results from the distortion of the structures holding the amino acid pool, and probably is related to the sensitivity of the pool to osmotic shock.

Some exploratory studies have been made on the reaction of hydroxylamine with the pool amino acids of *E. coli*. Hydroxylamine reacts rapidly with acyl phosphates, anhydrides, and halides to form hydroxamic acids; the corresponding carboxylates, amides, and peptides react very slowly. If the pool amino acids were present as activated forms such as acyl phosphates, a fairly efficient conversion to the corresponding amino hydroxamic acids would be expected in the presence of high concentrations of hydroxylamine. Experiments to test this possibility show very small yields of amino hydroxamic acids. In the two cases tested with great sensitivity, identifiable quantities of leucine and tyrosine hydroxamic acids have been observed. These quantities, however, correspond to a very small fraction of the native pool of these amino acids. In fact they were not present in an amount sufficient to supply the cells' requirement for protein synthesis for as long as 1 sec. The

experiments on the synthesis of nascent protein (12), however, indicate that the whole process from external sulfate to amino acid to protein is completed in about 3 sec. Thus the fact that activated amino acids are present in extremely small quantity does not, by itself, indicate whether or not they play a role in protein synthesis in the growing bacterial cell.

III. DISCUSSION OF THE MECHANISM OF POOL FORMATION

A. Introduction

The purpose of this section is to examine the implications of the large number of experimental observations presented in the previous sections. It appears necessary, in order to bring some clarity to a problem of this complexity, to start out by postulating models of the process. The discussion of the experimental observations in relation to the models allows the implications to be brought out more clearly.

The qualitative predictions of the relatively simple models considered here can be deduced easily. However, our knowledge of their quantitative predictions must depend on a fairly crude analysis. What are undoubtedly complex reaction sequences are taken to be single steps subject to the simpler equations of chemical kinetics. The coupling of the concentration process to the cell's energy supply, for example, is included in an almost purely symbolic way.

The state of the subject at this writing is that the two simple models that have been widely discussed, termed by Monod *permease* and *stoichiometric site*, both fail in a qualitative way to explain the presently known facts. Following a discussion of the reasons for these failures, a new model is proposed, which we have called the *carrier model*. The deductions from this model are consistent with practically all of the observations.

B. The Permease Model

The permease model is shown schematically in Fig. 28. Cohen and Monod state (8), "Obviously the actual mechanism of specific permeation must be more complex than the deliberately bare and abstract model we have set up." Further, they use the term "permease" nearly synonymously with "specific permeation mechanism." However, for reasons of clarity, in this

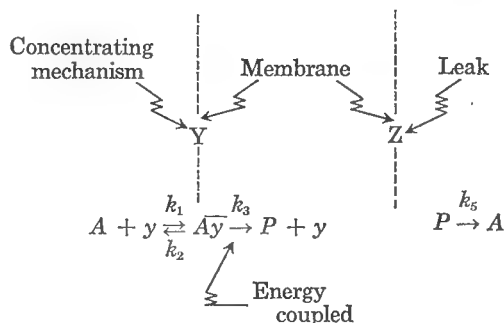


FIG. 28. *Permease model. Properties:*

- The bacterial cell is enclosed with an osmotic barrier which is highly impermeable to amino acids;*
- The impermeability is not absolute and leakage may occur, tending slowly to equilibrate the inside and outside concentrations;*
- Within the barrier exist proteins (the permease) capable of forming specific complexes with the amino acid;*
- The complex associates and dissociates reversibly with amino acid either inside or outside the barrier, and catalytically activates the equilibration of internal and external concentrations;*
- When coupled to an energy donor, the internal association reaction is, in effect, inhibited, and amino acid accumulates inside the cell;*
- As the internal concentration rises, the non-specific leakage increases until its rate balances the rate of accumulation at equilibrium;*
- The amino acid is presumed to be in a free state within the cell.*

discussion the term "permease model" will be restricted to a model with the essential features listed in Fig. 28. The list has been abstracted from (8) and is written for the case of an energy-dependent amino acid-concentrating system.

The permease model accounts for a great many of the observations regarding the concentration of substances by bacteria. There is no need here to repeat the extensive review of the evidence given in (8). It is sufficient for the moment to say that the evidence clearly shows that there are stereospecific sites which act as catalysts for the concentration of compounds by the cell. In other words, there are sites which participate in the early steps of the process but do not, by themselves, hold the compounds in the cell. Also some evidence indicates that the catalytic sites are protein in nature. Such sites will certainly have to be retained as a part of any adequate model for the pool-forming process.

TABLE 6. *Relationship of properties of the pool to the models*

Observation	Score ^a	
	Permease model	Carrier model
Formation and maintenance:		
1. Glucose required for formation.....	+	+
2. Glucose not required for maintenance.....	—	+
3. Pools formed slowly at 0 C.....	—	+
4. Pools maintained at 0 C.....	—	+
5. Pool maintained at 25 C in absence of either the amino acid or glucose.....	—	+
6. Pool size versus concentration not Michaelis.....	—	+
7. Initial rate of formation not proportional to pool size.....	—	+
8. Small pools not generally influenced by other amino acids but large pools are suppressed.....	—	+
9. Evidence for catalytic site in general.....	+	+
10. Pools may be very large.....	+	—
Exchange:		
11. Exchange occurs in addition to steady flow through the pool.....	+	+
12. Rapid exchange occurs in absence of glucose or at 0 C.....	—	+
13. Fast and slow components in exchange at 0 C.....	—	+
14. The 0 C exchange rate saturates at low external concentration.....	—	+
15. The 0 C exchange rate increases with pool size.....	—	+
Osmotic behavior:		
16. Pools removed by sudden reduction in osmotic strength.....	+	(+) ^b
17. Pools immediately re-formed after removal by shock.....	+	+
18. Different pools removed to different extent by shock.....	—	(+) ^b
19. Maximum pool size increases with osmotic strength of medium.....	—	—

^a A + sign indicates that the model satisfactorily explains the observation. A — sign indicates that there is a contradiction or that a modification may be required by the experimental evidence.

^b Assuming that the sites may be osmotically sensitive.

The permease model predicts a number of features of the concentration process which are contradicted by the evidence. In order to visualize these contradictions, we will first consider the way in which a pool is formed according to the permease model, and then discuss the experimental observations listed in Table 6.

An external amino acid first forms a complex with the permease (*y*) in the osmotic barrier. In the presence of an energy supply, the complex dissociates on the inside of the barrier. The free amino acid within the cell may then pass out of the cell by means of a nonspecific leak (*z*). Thus there will be a continuous circulation of amino acid through the pool at equilibrium when the two rates balance. For a given external concentration, the pool size will be determined by the rate at which the permease can pump amino acid into the cell. At the external concentration at which this rate saturates, the pool size will saturate.

The observations (Table 6, items 1 and 2) that glucose is not required to maintain a large internal concentration but is required to form a pool at normal rates clearly are not consistent with the above description of the pool-forming process. Similarly, the permease model fails to explain the observation (items 3 and 4) that, while pools are formed very slowly at 0 C, pools formed before chilling are maintained indefinitely (if not too large a size). From the point of view of the permease model, these results imply that when glucose is exhausted or the temperature is reduced to 0 C, not only is the active permeation process suppressed, but the "passive" leak is also suppressed to an *identical degree*. Further, the pool is maintained at 25 C in the absence of the amino acid, whether or not glucose is present. Thus, item d in the list of properties of the permease model is contradicted.

These observations clearly establish that a passive leak, as measured by pool maintenance

experiments, is very much too small to control the steady-state pool size by balancing against a simple active input process. They imply either that the leak must be made a rather strange function of the environmental conditions or that a more sophisticated active process must be considered. As will be illustrated in the discussion of the carrier model, a self-suppressing input mechanism seems to provide a more direct and satisfactory explanation of the empirical data.

The evidence on the exchange between external and pool amino acids summarized in items 12, 13, and 14 of Table 6 raises further difficulties for the model. Exchange under normal conditions, for example at 25 C, is expected, owing to the circulation through the pump and leak. The measured rate of exchange is comparable to the initial formation rate, as the model predicts. However, the high rates of exchange in the absence of glucose or at 0 C are not predicted. It might be suggested that the complex Ay can exchange with both A and P , at a rate higher than the actual association or dissociation in the absence of energy. The fact that the rate of exchange (at 0 C) saturates at a low concentration would suggest that the permease itself is saturated. In addition, some property of the permease must limit the rate of exchange. This is perhaps reasonable, since the complex cannot be exposed on the inside and outside of the membrane at the same moment. The permease model has been given a minus score in Table 6 for items 12, 13, 14, and 15 since an exchange mechanism must be added. In fact, this mechanism would have to be specified in detail in order to make an adequate comparison with the observations.

Item 12 shows clearly that the pool has more than one component. This argument has been given in detail in the experimental section on exchange. The evidence summarized in items 6, 8, and 18 also strongly supports this conclusion. From the point of view of the permease model, one might say that the cell has several pool-containing compartments separated by osmotic barriers, each containing an appropriate permease, but this is not a pleasant prospect. It seems much more likely that at least part of the pool is not in free solution within the cell, as will be discussed below.

The observation (item 6) that the pool size, far below saturation, does not rise in proportion

to the concentration could be taken to indicate that there is more than one component in the pool or that there are permeases with a variety of affinities for a given compound. For a number of reasons, this item cannot be considered a crucial argument against the permease concept. However, since the permease model we are discussing does not predict such a result, it is given a minus score for item 6.

The initial rate of pool formation (item 7) at the time the amino acid is supplied shows a much smaller variation with concentration than does the pool size. In fact, it saturates at a relatively low concentration. A similar conclusion can be drawn from the observation that a low concentration of isoleucine will block valine incorporation (Fig. 7). Both these observations show that the catalytic site for pool formation saturates at a concentration far below that at which the pool itself saturates. For this reason, these observations supply good evidence for the existence of a catalytic site. A characteristic of the permease model is that the circulating flow is proportional to the pool size. This circulating flow is, in turn, identical with the initial rate of pool formation. The failure of this proportionality further supports the conclusion that the pool is maintained by mechanisms other than the balance between a rapid active process and corresponding rapid leak.

It is clear from the above discussion that the simple permease model is inadequate because it fails to agree with experimental data in a number of ways. In addition, there is a philosophical objection to the model as written. This may be seen by noting that P is the same material as A , except that it is on the other side of the barrier. Why, then, don't P and y interact with the same rate constants (k_1 and k_2) as A and y ? Of course, the energy-coupled reaction (k_3) may occur only inside the cell because the energy carrier may be so localized. Now it might be suggested that if the energy-coupled reaction (k_3) is fast enough it will be so dominant that the ordinary reactions (k_1 and k_2) could be neglected inside the cell. However, note that the input rate is not greater than k_1yA . Therefore k_3Ay is also not greater than k_1yA . But k_1yP is much greater than k_1yA simply because P is ordinarily much greater than A . Thus it is seen that this philosophical objection is hardly pedantic, and one in fact has assumed the existence

of some rather tricky means of distinguishing the performances inside and outside the cell. This mechanism really is the key to producing the desired behavior, and the failure to display it explicitly simply avoids the whole question. In this sense the model really is not a scientific model at all. If we are to say that the materials inside the cell are somehow physically or chemically different from those outside, doesn't the real elucidation of the problem lie in explaining the nature of this difference?

From the experimental side, it is again worth noting that, to explain the relationship between pool sizes, loss rates, and initial formation rates, it would appear necessary to postulate a mechanism whose details provide that the size of the pool is not determined solely by the loss rate increasing until it equals the input, but rather by a heavy contribution from the input rate being lowered as the pool increases in size. In other words, the pool should inhibit its own formation.

One can postulate detailed mechanisms which meet both the philosophical objection to the simple model and the inhibiting requirement mentioned above. With such improvements the permease model becomes more satisfactory, but in order to make it fit the full variety of the experimental facts, even greater complexity and sophistication are apparently required. It seems out of place in this review to illustrate the possibilities of a number of models of increasing complexity. Suffice it to say that it may be possible to add enough special features to make a reasonably satisfactory model in which the elements of the simple permease model may perhaps still be recognized. In particular, the crucial part played by the osmotic barrier would presumably still be dominant in such an extended model.

C. The Stoichiometric Site Model

The central feature of any "site" model is that the amino acid is held by association with the macromolecules of the cell. For the moment, the nature of this association is unspecified, and it is presumed that the osmotic barrier which may exist near the surface of the cell is of minor importance in the maintenance of the pool. The simplest possible model of this type is shown schematically in Fig. 29.

It is immediately obvious that this model is unsatisfactory, since it does not explicitly contain a step equivalent to the catalytic site. Fur-

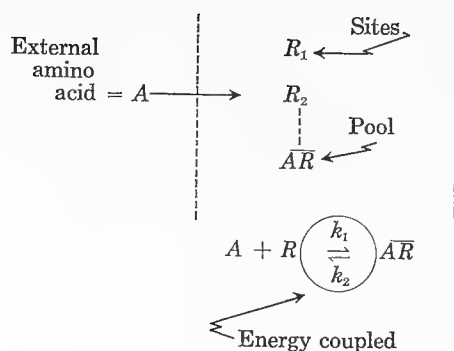


FIG. 29. Stoichiometric site model

ther, the rate of pool formation should be proportional to the amino acid concentration and the number of unoccupied sites.

In order that the pool size increase with the external concentration (at low concentrations), there must be a process by which the site-amino acid complexes dissociate. The fraction occupied would then be determined by a balance between the rate of formation and dissociation. The rate of dissociation, however, would have to be energy-dependent, in order that the pool be maintained as implied by items 2, 3, 4, and 5. Since all of these difficulties can be resolved together, we shall pass immediately to the discussion of the "carrier" model.

D. The Carrier Model

The carrier model is consistent with practically all of the known facts concerning amino acid pools. We have been led to postulate its central features by the failure of the previously discussed models.

The fact that pools are maintained under adverse conditions where they might be expected to leak out, combined with other evidence, has led us to include sites as the major mechanism for maintaining the pool. The strong evidence that a catalytic site participates in pool formation has led us to include such an intermediate step in the formation of the site-amino acid complex (pool). We have, therefore, postulated that the catalytic site is part of a molecule of moderate molecular weight termed the "carrier." The carrier molecule is assumed to be large enough to form a stereospecific complex with the amino acid, but still small enough to diffuse within the cell with some freedom. The mobility of the carrier is necessary, since there are few carriers

to transfer amino acids to the many pool-holding sites.

A schematic diagram of the carrier model is shown in Fig. 30, along with equations indicating the reactions proposed. The listed properties of the model have been specified quite sharply so that the deductions may be quantitatively analyzed. It cannot be ruled out that certain "forbidden" processes, such as exchange between free amino acid and site-associated amino acids, proceed at slow rates. Further, the evidence is insufficient to specify which of the two processes is actually coupled to an energy donor.

According to this model, the pool is formed in the following way. An external amino acid diffuses into the cell and collides with an unoccupied carrier. A complex with the stereospecific carrier is formed (\overline{AE}); the complex diffuses through the cell and collides with an unoccupied site. In a reaction coupled to an energy donor, the amino acid is transferred from the carrier to the site. In turn, an unoccupied carrier may collide with an occupied site and remove the amino acid. The evidence at the moment does not specify whether or not the reverse reaction is also energy-requiring.

As the pool rises, the reverse reaction ($\overline{AR} + E \rightarrow \overline{AE} + R$) reduces the quantity of free carrier (E). Thus, the rate of formation of carrier complex with free amino acid falls until it equals the rate required for protein synthesis. This is then the steady state.

With reference again to Table 6, the deductions from this model will now be compared with the observations. Item 1 is obvious, since the energy requirement has been built in. Two distinct properties of the model lead to maintenance of the pool when formation is suppressed (items 2, 3, 4, and 5). We could assume the reverse reaction ($\overline{AR} + E \rightarrow \overline{AE} + R$) to be energy-dependent, and then clearly the pool would be maintained in the absence of glucose. Alternatively, we are at liberty to choose a small value for the constant, k_2 , without influencing any other properties of the model, and therefore the loss rate under all conditions (short of damaging the cell) can be set as low as necessary. The choice of a small k_2 simply means that the carrier (or catalytic site) has a high affinity for the amino acid, and thus the amount of carrier complex will saturate at low concentrations. This is consistent with the saturation of the

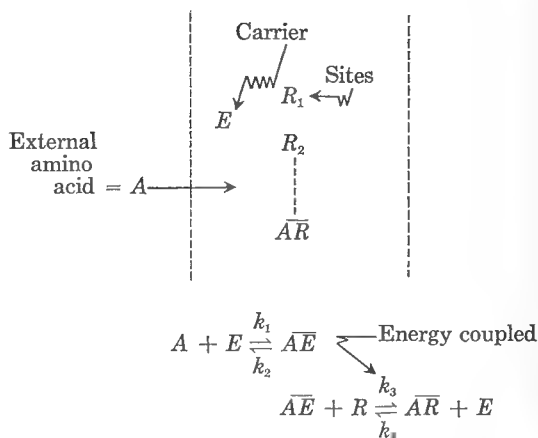


FIG. 30. Carrier model. Properties:

- a) The cell contains a small quantity of mobile stereospecific carriers which freely form complexes (\overline{AE}) with amino acids, without participation of energy donors;
- b) The cell also contains a relatively large quantity of nonmobile groups (the sites) which form complexes (\overline{AR}) with the amino acids;
- c) The site complex \overline{AR} can only be formed by a reaction with the carrier-complex \overline{AE} and this reaction is coupled to an energy donor;
- d) Exchange may occur between the site-associated amino acids and those associated with carriers, without coupling to energy donors;
- e) Exchange also occurs between free amino acids and carrier-associated amino acids, but not between free amino acids and site-associated amino acids;
- f) There are several classes of sites, some stereospecific and some nonspecific;
- g) There may be an osmotic barrier near the surface of the cell and "free" amino acid may not diffuse through the protoplasm at the same rate as in water, but the formation of the carrier complex nevertheless occurs at a sufficient rate, without the participation of an energy donor.

exchange rate (item 14) and of the formation rate (item 7) at low external concentrations.

The evidence on exchange between pool and external amino acids led to the postulation of the exchange processes described in items d and e in Fig. 30. (In this connection one might consider the discussion at the end of Part I-H). These processes are sufficient to explain all the observations on exchange (items 11, 12, 13, 14, and 15 of Table 6). Saturation of the exchange rate at low external concentrations is predicted if the natural assumption is made that the exchange rate between free amino acid and carrier

is more rapid than the exchange rate between the amino acids of the carrier complex and the site complex. Thus, when exchange is studied with labeled amino acid, the specific radioactivity of the amino acid associated with the carrier complex would always be close to that of the external amino acid. The amount of the carrier complex is saturated under the conditions of the experiment at 0 C. The collisions between the carrier complexes (constant specific activity and quantity) and the site complexes would control the exchange. The rate of exchange would, therefore, be independent of the external concentration and proportional to the pool size.

In the mathematical appendix, the carrier model is examined in some detail. Allowance has been made for two different kinds of sites. In this model it is important to account for the utilization of amino acid for protein synthesis, for the native pool, and for the competition between internally synthesized and exogenous amino acid if quantitative evaluation of the model is to be made. The constants of the model (for proline in *E. coli*) which give a relatively good quantitative correlation with the data are presented in the appendix. These constants were obtained from experiments on pool sizes and rates of formation. It is very pleasing that so much information on the details of the concentration relationships and the competition between synthetic processes and concentrating processes can be encompassed in one conceptually simple model. One interesting consequence of the treatment is that the two sets of sites correspond to two components of the pool, one of which has a saturation value 20 times the other. The larger component has a half-saturation value of external concentration which is 100 times that of the smaller and, as a consequence, is only dominant at the higher end of the concentration range. It is very satisfying that the existence of two components of the pool of such different characteristics provides qualitative agreement with conclusions deriving from experiments on exchange and osmotic shock.

The removal of the pool by osmotic shock (items 16, 17, and 18) is not an obvious prediction of the carrier model. The simplest explanation, from the point of view of this model, is that the sites themselves are temporarily affected during a transient period of distention of the cell. The implication is that the macromolecules

on which the sites are located are temporarily distorted in such a way that the affinity of the site for amino acid is drastically reduced. This could result from a direct change in the hydration of the macromolecule itself or from a mechanical coupling of the macromolecule to major cell structures. It must be pointed out that a semipermeable membrane is not necessary for osmotic phenomena to occur. An ion exchange column (Dowex 50, 2% cross-linked) will undergo striking volume changes when sucrose solutions of different concentrations are passed over it.

From this point of view, some interesting speculation about items 19 and 10 may be indulged in. The size of a very large pool, which is nonspecific, is roughly proportional to the osmotic strength of the medium. Thus, the maximum pool is somehow related to the osmotic balance of the cell, although even the largest amino acid pools account for only a small fraction of the total osmotically active material that may be released from the cell. It may be suggested that there exist nonspecific associations between the amino acids and the dense protoplasm (25% dry material) of the bacterial cell. The maximum quantity of amino acid in such an association might decrease with increasing hydration of the protoplasm. No such associations are observed in relatively dilute protein solutions or in disrupted suspensions of cells. However, a carrier or energy donor present in the living cell might be a necessary condition in the formation of such an association.

The fact that different pool compounds are removed in varying degrees by a given osmotic shock (item 18) probably reflects the differences in sensitivity of site complexes.

Finally, it is difficult to leave the discussion of this model without some speculation on the nature of the carrier. The properties required of the carrier, for its function in this model, are that it be a large enough molecule to form a stereospecific association with an amino acid and, on the other hand, that it be small enough to diffuse with some freedom within the cell. Further, it must have a high affinity for free amino acid and be able to give up amino acid freely to form a site complex. In turn, unoccupied carriers must be able to accept amino acids from the site complexes.

At least two possible candidates for the carrier are known at present. The lipid-amino acid

complex discovered by Hendler (10) in the hen oviduct has been observed in *E. coli*. The quantity and rapidity of labeling of such complexes in tracer experiments are consistent with the possibility of their function as carriers in the sense of this model. However, the molecular weight is unknown. The soluble ribonucleic acid amino acid complex discovered by Hoagland also occurs in *E. coli* in very small quantities. However, there is no indication (1) that the rate of turnover of the amino acid in this complex is fast enough to carry out the function of the carrier.

A crude lower limit on the amount of carrier present in the cell can be set from its turnover number and rate of diffusion. At the maximum rate of proline pool formation, there are 40,000 molecules entering the pool per sec per cell. The time required for a small molecule such as proline to diffuse $1\ \mu$ is about 1 msec. The mean distance over which the carrier must diffuse between taking up an amino acid and delivering it to a site is much smaller than this. However, the molecular weight is probably much larger, and the diffusion constant is probably much smaller, in the protoplasm than in water. Thus, the turnover number probably would be considerably less than 1,000 per carrier per sec, and therefore the number of carriers would be greater than 40 per cell. If the number of carriers were as small as this, it would certainly be difficult to observe the carrier complex directly.

The properties of "cryptic" mutants have had an important place in discussion of bacterial-concentrating mechanisms (7). They have not been mentioned so far, since we have limited ourselves to amino acid-concentrating systems. For our purposes, the observations may be summarized as follows. There are strains of *E. coli* (y^- , z^+ , i^-) which contain large quantities of the enzyme, β -galactosidase, but will not utilize lactose or concentrate galactosides. The enzyme is fully active in preparations of these cells treated with toluene. Undamaged cells will only split the test-substrate (*o*-nitrophenylgalactoside, ONPG) at low rates when it is supplied at high concentrations. Other strains which utilize lactose contain both the enzyme and an operative concentrating system. The enzyme in these cells will split ONPG at high rates whether treated with toluene or not and when the concentration

of galactosides has been blocked by metabolic inhibitors.

The fact that the mechanism for the concentration of galactosides and the enzyme, β -galactosidase, are controlled by distinct genetic loci is consistent with the carrier model. Clearly the carrier, the sites, and the enzyme would be three distinct elements in the cell. However, in the carrier model no osmotic barrier limits the rate of entry of substrate. What, then, limits the rate of splitting of the substrate (ONPG) by the enzyme present in the cryptic mutant (y^- , z^+ , i^-)? An additional hypothesis is necessary: In the organized cell, the free substrate does not have full access to the enzyme, but when associated with the carrier, it can reach the active site and be attacked at maximal rate. In defense of this hypothesis, it may be pointed out that there are a large number of examples of enzymatic reactions which are suppressed or absent in whole cells but occur at high rates in disrupted cell preparations. In some cases, the suppression has been attributed to an impermeable barrier, but there are a number of examples for which the substrate is known to be present in the cell, and such an explanation is clearly invalid. The fact that (in y^+ strains) metabolic inhibitors block the concentration process but do not reduce the rate of splitting of ONPG further implies that the carrier complex is formed rapidly without the participation of energy donors.

Support for the carrier model also comes from experiments on the concentration and utilization of nucleic acid bases by *E. coli* (11; M. Buchwald and R. J. Britten, *unpublished data*). The experimental facts, however, differ in almost every conceivable way from those relating to amino acid concentration processes.

The bases are almost instantaneously converted to nucleotides. The rate of incorporation of an exogenous base into ribonucleic acid (RNA) may reach, within 5 sec, half the total rate of incorporation of the corresponding residue into RNA. Thus there exists a "by-pass" around the large pool of nucleotides stored in the cell. Further, the size of the nucleotide pool is independent of the external concentration of the base; i.e., the nucleotide pools are not expandable. The maximum rate of uptake of the base by the cell is not larger than the requirement for RNA synthesis. Nevertheless the cells rapidly take up bases at low concentrations (10^{-7} M).

It appears that simply by changing the values of the reaction rate constants, the predictions of the carrier model may be altered to agree with the observations of the incorporation of bases into nucleic acid. Specifically, if k_3 is very much greater than k_4 (Fig. 30), then there will be few free sites (R), and the pool will always be near the saturation level. Further, the small quantity of free sites reduces the rate of transfer of the nucleotide from the carrier complex to the storage sites. Thus the rate of entry into RNA may be comparable and the "by-pass" is explained. Of course, if the pool is always near saturation, the rate of entry into the cell cannot be greater than the rate of incorporation into RNA.

Quantitative evaluation of the rate constants for the incorporation of nucleic acid bases has not yet been carried out; it appears obvious, however, that the carrier model is capable of explaining qualitatively all of the known features of the process. These features would be quite difficult if not impossible to interpret, if it were assumed that the nucleotides were in free solution within the cell. The very existence of the by-pass demonstrates that at least a part of the pool is organized in the cell in a very different way from the large pool of nucleotides.

E. Conclusion

In this paper we have outlined the rather diverse set of experimental observations of the amino-acid-concentrating processes in *E. coli*. Any satisfactory model of these processes must be formulated with all of this information in mind.

We have reviewed the simple "permease" and simple "stoichiometric site" models and pointed out their failures. Then we have developed the "carrier" model in some detail and demonstrated that it can be made to correlate almost all of the data in a highly satisfying manner.

While it is tempting to do so, it should not be asserted that the function of sites and carriers has been rigorously proved or that the function of an osmotic barrier has been demonstrated to be unimportant. All that can be said is that the equations derived from the carrier model accurately describe the experimental data. Possibly these equations are not unique to that model. Any model which gave essentially the same equations would also be satisfactory, and one which gave essentially different equations, clearly unsatisfactory.

The permease model might be elaborated to remove some of its difficulties. Part of such an elaboration would have to consist of specifying the mechanism by which asymmetry in reaction constants on the two sides of the barrier is obtained. This mechanism might introduce additional features which would modify greatly the properties of the permease model. We must ask whether any mechanism can be proposed which has the necessary properties, if the pool is assumed to be free in solution. The minimal requirements are rapid pool formation; slow loss in absence of glucose, or amino acid, or both; increase of pool with external concentration until saturation is reached; and lack of proportionality between formation rate and pool size. If an exhaustive search does not yield a model with these properties, then there would be no reasonable alternative but to assume that the amino acid pool is not in free solution within the cell. In this connection it should be remembered that experimental data conclusively demonstrate that the pool has at least two components, so that if we are to assume that the pool is unbound amino acid, we must introduce at least one more osmotic barrier. Possibly it would be necessary to resort to a combination of a site and barrier.

Of course, there are many problems which deserve further investigation. The meaning of the term "free in solution" needs to be examined both experimentally and theoretically. A 25% solution of protein, RNA, etc., organized in subtle ways, is certainly an unusual solvent from a chemical point of view. The activity of the amino acids might be strikingly depressed. The results of such a study would have very broad implications for other processes in living cells.

Finally, a more detailed experimental study of the rates of loss and exchange is needed. The study of these phenomena with pools of several amino acids and other compounds, such as galactosides, would supply quantitative information that might be helpful in deciding among the alternative mechanisms.

With present knowledge, alternative interpretations of the concentration process in bacteria still remain possible, in spite of the large amount of experimental evidence which has given insight into many aspects of the process. The simplicity and the degree to which individual steps may be understood, from a chemical point of view, differ among the various

models. It is for the future to decide which of the alternative approaches will be most useful.

IV. APPENDIX: MATHEMATICAL ANALYSIS OF THE MODELS

A. Method of Calculation

For the purposes of making these chemical kinetic calculations, it has been presumed that the main steps postulated for each of the models behave as simple molecular reactions. In other

words, in the reaction, $A + R \xrightleftharpoons[k_2]{k_1} \overline{AR}$, the rate of formation of the complex is simply k_1AR , and the rate of dissociation is $k_2\overline{AR}$. The possibility of intermediate steps or other complicating factors has been ignored. Such an approach can only be defended by its usefulness in supplying insight into the proposed models and by the agreement of the results of the calculations with experimental data.

In much of the analysis which follows it has been presumed that the external amino acid concentration is constant throughout the period in which the pool is built. In other words, of the total amino acid present in the system, only a small fraction is taken up into the pool. This situation is easily achieved experimentally at low cell densities. Correction must be made for changing concentrations in the evaluation of experiments at higher cell densities.

The system being examined in this analysis is bounded by the external surface of the cell, and all of the internal concentrations are expressed per unit volume of wet cells. When the external concentration remains constant, the volume of medium surrounding the cells does not enter the equations. In general, the calculation should be restricted to a region, of unknown volume, smaller than the whole cell. If it were possible to define the boundaries of the system to coincide with the actual reaction volume, the effective internal concentrations would be higher. Thus, some of the derived rate constants are subject to this uncertainty as far as their absolute values are concerned.

For simplicity, the utilization of pool amino acid for the synthesis of protein has not been taken into account in the analysis of the permease model. This approximation is justified for this model, since there is always a large flux into and out of the pool through the permease and leak. At high concentrations the flux is 10 times the

rate of utilization for protein, and at the lower concentrations studied the flux is 3 times the protein rate. Thus, at low concentrations, the predicted pool values would be lowered by taking the protein rate into account. Even without this correction, however, the predicted values fall well below the measured values. For the carrier model, on the other hand, the situation is quite different. In this case the rate of flow of amino acid into the pool is strongly suppressed as the pool rises toward its steady value, and therefore the flow into protein cannot be ignored.

The following symbols have been used to represent the concentration per unit cell volume of the various species entering the reactions:

$$\begin{aligned} y &= \text{free permease} \\ \overline{Ay} &= \text{permease-amino acid complex} \\ Q = y + \overline{Ay} &= \text{total permease} \\ P &= \text{pool amino acid} \\ E &= \text{free carrier} \\ \overline{AE} &= \text{carrier-amino acid complex} \\ z = E + \overline{AE} &= \text{total carrier} \\ R &= \text{free site} \\ \overline{AR} &= \text{site-amino acid complex (pool)} \\ \pi = R + \overline{AR} &= \text{total sites of one class} \\ \rho &= \text{volume fraction in cells.} \end{aligned}$$

In addition, A represents the external amino acid concentration per unit volume of medium; k_1 , k_2 , etc. represent the rate constants for the various reactions; and α represents the constant rate of utilization of pool amino acid for the synthesis of protein.

B. The Permease Model

The reactions postulated for the permease model are shown in Fig. 28. The rate of change of the amount of permease complex is simply the sum of the rates of formation and dissociation in the three reactions indicated by k_1 , k_2 , and k_3 .

$$\frac{d\overline{Ay}}{dt} = k_1Ay - k_2\overline{Ay} - k_3\overline{Ay} \quad (1)$$

Similarly, the rate of change of the pool can be written as

$$\frac{dP}{dt} = k_3\overline{Ay} - k_5P \quad (2)$$

The total quantity of permease is

$$Q = \overline{Ay} + y \quad (3)$$

When the pool has reached a steady value, both

equations 1 and 2 are equal to zero. Therefore, from equations 1 and 3,

$$\overline{Ay} = Q \frac{A}{\frac{k_2 + k_3}{k_1} + A} \quad (4)$$

Substituting this result into equation 2,

$$P = \frac{k_3 Q}{k_5} \cdot \frac{A}{\frac{k_2 + k_3}{k_1} + A} \quad (5)$$

Thus the pool size, as expected, follows a classical adsorption isotherm.

The initial rate of formation of the pool is most easily evaluated by making the so-called steady-state approximation. The amount of permease complex changes slowly or not at all after it almost instantaneously reaches its steady value. Thus, equation 1 may be taken to be equal to zero; therefore, equation 4 again results. Equation 4 is substituted in equation 2, taking the leak rate, $k_5 P$, to be negligible at early times (P is small).

$$\left(\frac{dP}{dt}\right)_{P=0} = k_3 \overline{Ay} = k_3 Q \frac{A}{\frac{k_2 + k_3}{k_1} + A} \quad (6)$$

Thus, by comparing equations 5 and 6, it is seen that the initial rate of formation is proportional to the pool size that is finally achieved for systems of low cell density.

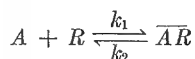
The initial rate of loss from the pool when the external amino acid is removed also is easily evaluated. Since in this model the permease complex, \overline{Ay} , can only be formed from outside, $\overline{Ay} = 0$ when $A = 0$. Therefore,

$$\left(\frac{dP}{dt}\right)_{A=0} = -k_5 P \quad (7)$$

Thus, by comparing equations 5, 6, and 7, it is clear that the initial rate of loss after the amino acid is removed is identical with the initial rate of formation when the amino acid was originally added for systems of low cell density.

C. The Stoichiometric Site Model

The reaction for this simple model is



Also,

$$R + \overline{AR} = \pi \quad (1)$$

The rate of change of the pool can be written as

$$\frac{d\overline{AR}}{dt} = k_1 AR - k_2 \overline{AR} \quad (2)$$

When a steady pool is achieved, equation 2 equals zero; by using equation 1,

$$\overline{AR} = \pi \frac{A}{A + k_2/k_1} \quad (3)$$

The rate of formation when the pool is zero, $(d\overline{AR}/dt)_{\overline{AR}=0} = k_1 A \pi$, does not saturate with increasing external concentration.

The rate of loss when $A = 0$ is

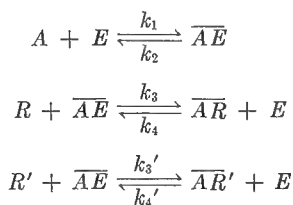
$$\left(\frac{d\overline{AR}}{dt}\right)_{A=0} = -k_2 \overline{AR}$$

Therefore, the rate of loss is proportional to the pool size. Using equation 3, we see that, at small external concentrations, the rate of loss equals the initial rate of formation. At large concentrations it is smaller.

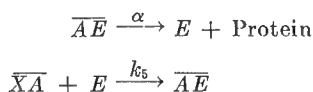
D. The Carrier Model

The reactions postulated for this model are shown in Fig. 30. The experimental evidence has shown that loss from the pool occurs only at low rates, and this fact is reflected in the model. As a result, when a steady pool is formed (in growing cells), the rate of uptake into the cell is approximately balanced by the rate of utilization for protein synthesis. Thus, the utilization for protein synthesis must be taken into account. Further, when the external concentration is small, the cell synthesizes a significant part of its amino acid requirement. There follows a full set of reactions including these processes, with two distinct sets of pool-holding sites.

Concentrating mechanism.



Synthesis and utilization.



The last reaction represents a possible scheme for the cell's control of the rate of amino acid

synthesis. It is proposed that amino acid is synthesized and remains associated with the final synthetic enzyme site, forming the complex, \overline{XA} . When a free carrier collides with this complex, the amino acid is taken up by the carrier. Essentially instantly another internally synthesized amino acid is formed, so that the concentration of \overline{XA} is constant. The rate of flow of amino acid from the synthetic site is therefore $k_5\overline{XAE}$, which is written as βE for simplicity. Thus, in this model the concentrations of carrier complex and free carrier control not only the rate of incorporation of amino acid and the quantity of pool but also the rate of internal synthesis of amino acid.

The total quantities of carrier and pool holding sites are both limited.

$$E + \overline{AE} = z \quad (1)$$

$$R + \overline{AR} = \pi \quad \text{and} \quad R' + \overline{AR}' = \pi' \quad (2)$$

The rates of change of the carrier complex and site complex (pool) are as follows,

$$\frac{d\overline{AE}}{dt} = k_1 EA - k_2 \overline{AE} + k_4 \overline{E\overline{AR}} - k_3 \overline{RAE} \quad (3)$$

$$+ k_4' \overline{E\overline{AR}'} - k_3' R' \overline{AE} + \beta E - \alpha$$

$$\frac{d\overline{AR}}{dt} = k_3 \overline{RAE} - k_4 \overline{ARE} \quad (4)$$

$$\frac{d\overline{AR}'}{dt} = k_3' R' \overline{AE} - k_4' \overline{AR}' E \quad (5)$$

and their sum is

$$\begin{aligned} \frac{d\overline{AE}}{dt} + \frac{d\overline{AR}}{dt} + \frac{d\overline{AR}'}{dt} \\ = k_1 EA - k_2 \overline{AE} + \beta E - \alpha \end{aligned} \quad (6)$$

When a steady pool exists, equations 3, 4, 5, and 6 are equal to zero. Under these conditions, the fraction of the carrier that is free may be calculated from equation 6 by using equation 1.

$$\frac{E}{z} = \frac{k_2 + \alpha/z}{k_1 A + k_2 + \beta} \quad (7)$$

The rate of synthesis of amino acid is

$$\beta E = \beta \frac{\alpha + k_2 z}{k_1 A + k_2 + \beta} \quad (8)$$

The net rate of flow of amino acid from the environment into the cell is

$$k_1 EA - k_2 \overline{AE} = \alpha \frac{k_1 A + k_2 - \beta k_2 z / \alpha}{k_1 A + k_2 + \beta} \quad (9)$$

The steady pool size may be calculated by setting equations 4 and 5 equal to zero and using equations 1, 2, and 7.

$$\begin{aligned} \overline{AR} + \overline{AR}' = & \frac{\pi}{1 + \frac{k_4(k_2 + \alpha/z)}{k_3(k_1 A + \beta - \alpha/z)}} \\ & + \frac{\pi'}{1 + \frac{k_4'(k_2 + \alpha/z)}{k_3'(k_1 A + \beta - \alpha/z)}} \end{aligned} \quad (10)$$

Equation 10 has the form of the sum of two adsorption isotherms in the higher concentration range, but automatically includes the native pool at the very lowest concentrations. Numerically it is essentially indistinguishable from a simple sum of the native pool and two classical adsorption isotherms.

The native pool formed when no supplemental amino acid has been added to the culture may also be calculated. Since all of the amino acid is internally synthesized in this case, $\beta E_0 = \alpha$. Setting equations 4 and 5 equal to zero and noting that, because the native pool is small, $R \simeq \pi$ and $R' \simeq \pi'$, we obtain

$$\begin{aligned} \overline{AR}_0 + \overline{AR}'_0 \simeq & \frac{k_3 \pi}{k_4} \left(\frac{z\beta}{\alpha} - 1 \right) \\ & + \frac{k_3' \pi'}{k_4'} \left(\frac{z\beta}{\alpha} - 1 \right) \end{aligned} \quad (11)$$

Also, since in this case $k_1 A_0 E = k_2 \overline{AE}$, the corresponding external concentration is

$$A_0 = \frac{k_2}{k_1} \left(\frac{z\beta}{\alpha} - 1 \right) \quad (12)$$

The initial total rate of incorporation of amino acid from the medium at the instant the amino acid is added may be calculated by using the steady-state approximation. The rate of change of the amount of carrier complex, \overline{AE} , is taken to be zero, after an extremely short period during which it rises to its slowly changing value. A numerical check with the constants listed in Table 7 has shown this approximation to be very good. Again, because of the small size of the native pool, we may put $R = \pi$ and $R' = \pi'$. Equation 3 then becomes

$$\begin{aligned} k_1 EA - (k_3 \pi + k_3' \pi') \overline{AE} \\ + (k_4 \overline{AR}_0 + k_4' \overline{AR}'_0) E \\ + \beta E - \alpha \simeq 0 \end{aligned}$$

TABLE 7. Numerical evaluation of constants for the carrier model: concentration of proline by *E. coli*

Parameter	Symbol	Numerical value
Data utilized:		
1. Quantity of sites, first component	π	8.0×10^{-2} mole/liter
2. A_k^a for sites, first component		1.0×10^{-4} mole/liter
3. Quantity of sites, second component	π'	4.3×10^{-3} mole/liter
4. A_k for sites, second component		1.0×10^{-6} mole/liter
5. Maximum total rate of incorporation		6.0×10^{-5} mole/liter \times sec
6. A_k for total rate of incorporation		1.5×10^{-6} mole/liter
7. Rate of utilization for protein synthesis		1.0×10^{-5} mole/liter \times sec
8. Native pool		2.0×10^{-4} mole/liter
9. Native concentration ratio		2.5×10^4
Derived constants:		
Arbitrarily chosen	z	1.0×10^{-5} mole/liter
From native pool (8) ^b	β	1.16 sec ⁻¹
From A_k for rate (6)	k_1	4.80×10^6 liters/mole \times sec
From native concentration ratio (9)	k_2	0.25 sec ⁻¹
From maximum rate (5)	$k_3 = k_3'$	59.3 liters/mole \times sec
From A_k , first component (2)	k_4	2.26×10^4 liters/mole \times sec
From A_k , second component (4)	k_4'	2.33×10^2 liters/mole \times sec

^a Represents the external concentration at which each parameter reaches one-half of its maximum value.

^b The experimental parameter listed is dominant in determining the constant, although the value is, of course, influenced by other parameters. Numbers in parentheses refer to the equations.

Using equation 1 and substituting \overline{AR}_0 and \overline{AR}_0' , we may solve for E and then write the initial total rate of incorporation from the environment.

$$k_1 A E \simeq \frac{k_1 A (k_3 \pi z + k_3' \pi' z + \alpha + k_2 z)}{k_1 A + \frac{z \beta}{\alpha} (k_3 \pi + k_3' \pi') + \beta + k_2} \tag{13}$$

In the experiments designed to measure the small leak rate, $k_2 A \overline{E}$, protein synthesis was inhibited, the pool was fairly large, and the external concentration was very low. Examination of the equations and numerical analysis show that under these conditions the amount of free carrier will be very small, and therefore the loss rate will be simply $k_2 z$.

E. Evaluation of the Rate Constants of the Carrier Model

Analysis of the experimental data. Figure 31 presents the best available data for the numerical evaluation of the constants of the carrier model for proline in *E. coli*. The results presented in this figure are principally those shown in Fig. 20, converted to chemical units. However, a more precise method for the calculation of the pool size has been utilized which influences somewhat the

values at low concentrations. In addition, an experiment at a very low concentration, which was carried out simultaneously, has been included.

When radioactive amino acid is added at low concentrations, it is diluted by internally synthesized amino acid. Thus, the radioactivity of the pool is not a direct measure of the pool size. However, the pool size may be calculated precisely if it is assumed that the pool is the source of amino acid utilized for protein synthesis and that the unlabeled, internally synthesized and the labeled amino acid are completely mixed in the pool. The rate of incorporation of labeled amino acid (\overline{AR}^*) into protein is

$$\frac{dp^*}{dt} = \frac{\overline{AR}^*}{\overline{AR}} \alpha$$

Thus

$$\overline{AR} = \frac{\alpha \overline{AR}^*}{dp^*/dt}$$

Here \overline{AR}^* and dp^*/dt are the measured radioactivity of the pool and rate of incorporation of radioactivity into protein. α , the total rate of utilization for protein synthesis, was evaluated

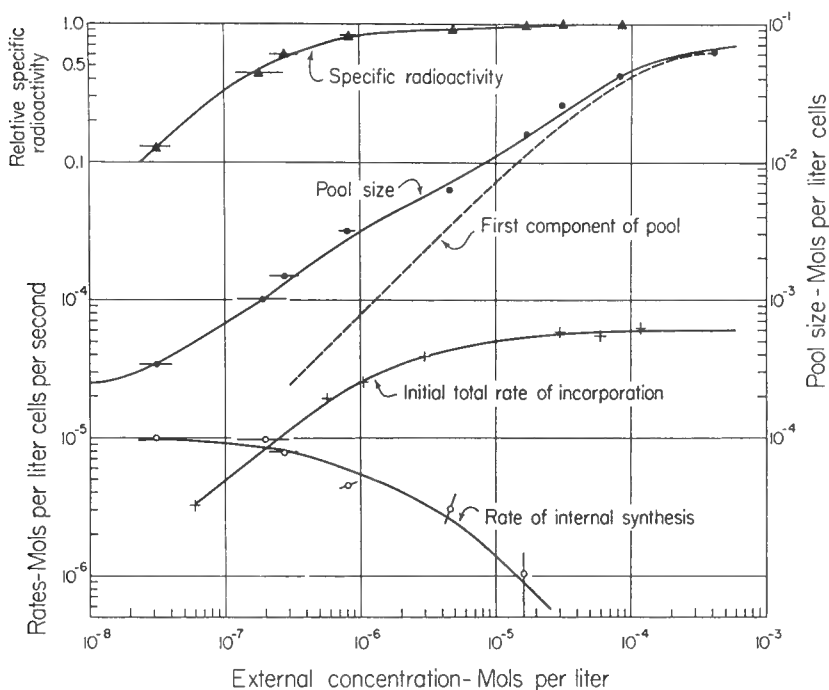


FIG. 31. Proline pool formation in *E. coli* at 25°C; log-log plot. The experimental points are derived from a set of eight simultaneous measurements of the time course of incorporation of radioactive proline, except for the point at $4.2 \times 10^{-4} M$, which is the average of several measurements of the saturation pool size. ▲, Ratio of specific radioactivity of the pool (at the time it reached its maximum value) to that of the added radioactive proline. ●, Maximum pool size reached versus concentration present at that time. ---, Prediction of the carrier model for the size of the major component of the pool alone. +, Initial total rate of incorporation of external proline. ○, Calculated average rate of internal synthesis of proline during the time required for the pool to reach its maximum value.

from several experiments at high external concentration where internal synthesis was completely suppressed. For Fig. 31, \overline{AR} was evaluated by means of this equation at the time the rate of change of the pool was zero, for each of the eight experiments. There remains an uncertainty in the external concentration present at this time, since internally synthesized amino acid may appear in the environment by exchange with the radioactive amino acid added. As a correction could not be made without a knowledge of the exchange rate, the probable limits of error due to exchange are shown by the horizontal bar through each point.

The curve drawn through the experimental points for the pool size is the sum of two adsorption isotherms and the native pool. This is the curve predicted by the carrier model (equation 10) for the constants listed in Table 7.

The next stage in the analysis is to calculate the rate of internal synthesis of amino acid. Both the radioactivity and the size of the pool are

known at the time the pool reaches its maximum value, and thus the specific radioactivity of the pool may be calculated. The upper curve on Fig. 31 shows the ratio of the specific radioactivity of the pool (at the time it reached its maximum value) to that of the originally added amino acid. The dilution observed at the lower concentrations arises from internal synthesis during the time required to build the pool and from the "native" pool initially present. (An examination of the ratio of internal to external concentration in the experiment at the lowest concentration shows that the external amino acid originally present in the culture makes but a small contribution to the dilution.)

A precise calculation of the average rate of internal synthesis during the time required to build the pool would require a knowledge of the exchange rate, since a certain amount of the internally synthesized amino acid appears in the environment by exchange. However, the calculation is possible for the case of zero exchange

and the case for which the exchange rate is so great that the internal and external specific radioactivities are always equal. By such calculations one can place limits on the rate of internal synthesis. The probable outside limits are plotted at their appropriate external concentrations and joined by solid bars.

For the two experiments at the lowest concentrations, the rate of synthesis was taken to be equal to the requirement for protein synthesis, and thus the native pool could be calculated. For each of these experiments the result was $2.0 \pm 1.0 \times 10^{-4}$ mole per liter of cells, and this value was used in evaluating the four cases at higher concentrations.

The final step in the analysis of the experiments is to calculate the initial rate of incorporation of exogenous amino acid. For this purpose the radioactivity remaining in the environment was plotted on semilogarithmic paper. For the first four cases (below 10^{-5} M external concentration), a straight line fitted the data accurately for times extending almost out to the time when the maximum pool size was reached. Thus an accurate value of the time constant (T) could be obtained, and the initial rate of incorporation, $-dA/dt$, was easily calculated from the relation

$$\frac{1}{T} = -\frac{1}{A^*} \frac{dA^*}{dt} = -\frac{1}{A} \frac{dA}{dt}$$

For the experiments at the four higher concentrations, the less accurate but essentially equivalent methods mentioned in Part II-I were utilized.

It is interesting from the point of view of the model that the external concentration decays exponentially, since

$$\frac{1}{\rho T} = -\frac{1}{\rho A} \frac{dA}{dt} = k_1 E - \frac{k_2 \bar{A} E}{A}$$

The small rate of loss observed when the external concentration was strongly reduced, and the high ratio of internal to external concentration observed in the experiment at the lowest concentration, both show that the second term, $k_2 \bar{A} E / A$, is negligible. Therefore, in the four experiments below 10^{-5} M, E , the amount of free carrier, is nearly constant with time. In the three experiments at the lowest concentrations, E in fact increases slightly as the maximum pool is approached. In the four experiments at the higher concentrations, E decreases as the maximum pool is approached.

Numerical evaluation of constants. The curve drawn through the experimental points for the pool size is the sum of the native pool and two adsorption isotherms with the constants shown in Table 7 (items 1, 2, 3, and 4). The first pair of constants, π and π' , are simply the saturation values of the two components.

The total quantity of carriers, z , has no influence on the predictions of the model as long as it is small. It has therefore been arbitrarily chosen to be 5% of the native pool or 6,000 carriers per cell. All of the derived constants are inversely proportional to z except π and π' .

Equations 10, 11, 12, and 13 each include several of the constants to be evaluated. The resulting set of simultaneous equations was solved by successive approximations. An approximate form was written down for each of the equations, eliminating unimportant terms. These equations were then numerically solved, and the resulting approximate constants were substituted in the complete equations. Since each of the experimental parameters dominantly affects a particular constant, it is a simple matter to adjust the constants (in proper sequence) until an accurate fit is obtained. Actually the system converges in one cycle.

There is no experimental evidence available which allows the rates of formation of the two components of the pool to be evaluated separately. Therefore, k_3 and k_3' were arbitrarily chosen to be equal.

With the exception of the native pool and native concentration ratio, (that is, the ratio of pool per liter of cells to external amino acid per liter of medium), the experimental parameters are known to be better than 20%. The determination of the native pool has been discussed above. The native concentration ratio was estimated from the experiment at the lowest concentration shown on Fig. 31. This experiment cannot be interpreted unambiguously because of the unknown rate of exchange between the pool (which is continuously being diluted by internal synthesis) and the external amino acid. The uncertainty in these numbers probably is a factor of 2 either way. The measurement of the rate of loss shown in Fig. 23 implies a lower value of k_2 , but this was performed with a different strain of *E. coli*.

The measured values of the rate of internal synthesis of proline have not been used in the

evaluation of the constants. The lowest curve shown on Fig. 31 was calculated from the carrier model by using the constants shown in Table 7. This calculation is for the average rate of internal synthesis during the period of pool formation for these particular experiments. For steady pool and constant external concentration, the model predicts a similarly shaped curve, which falls to half-value at 3.1×10^{-7} m.

The agreement with the experimental points probably is within the experimental error. This demonstrates the adequacy of the carrier model in terms of our present experimental knowledge.

It is interesting to note that if one calculates the size of the two components as indicated above for the exchange experiment shown in Fig. 18, then the exchange rate of each component is proportional to its size with a single proportionality constant.

V. ACKNOWLEDGMENT

In writing this article we have used freely the experiments and concepts of our colleagues at the Department of Terrestrial Magnetism, especially R. B. Roberts, D. B. Cowie, and E. T. Bolton. They have also liberally given guidance and advice. We are much indebted to the wife (B. H. B.) of one of us for assistance with the typing and editing.

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II.B.3 Protoplasts

(Reprinted from Carnegie Institution of Washington Year Book 56, pp. 131-133, 1957.)

E. T. Bolton and J. J. Leahy

PROTOPLASTS

The effects of lysozyme on the structure and function of E. coli. In order to study the macromolecular composition of the bacterial cell by physicochemical methods, such as ion exchange and ultracentrifugation, it is necessary to disrupt the cell and release its contents. The use of the enzyme lysozyme, together with osmotic shock, has proved an efficient way of breaking *E. coli*. The conditions for lysis and physical studies of the lysates are described below. Lysozyme and osmotic shock may also be used to inflict controlled damage upon *E. coli* and thus provide altered cells, spherical forms which are usually called "protoplasts." Study of these forms should prove helpful in evaluating what structures of the cell must remain intact in order for macromolecule synthesis to occur at normal rates. Metabolic studies on the spherical forms are described in the following paragraphs.

Lysis due to lysozyme treatment and osmotic shock. Cultures of *E. coli* growing or resting in various media (C medium, C medium diluted with 9 volumes of water, or C medium containing 18 per cent sucrose) show no response to added lysozyme (100 $\mu\text{g}/\text{ml}$). They neither lyse, show an impaired growth rate (15°, 23°, or 37° C), nor exhibit morphological change visible in the phase-contrast or dark-field microscopes. When such cultures are harvested, washed free of excess lysozyme by centrifugation, suspended in 0.5 M sucrose, and suddenly diluted with 10 volumes of distilled water, the bacteria continue to maintain their normal rodlike structure and upon subculture grow as well as the corresponding control (lysozyme-

omitted) cultures. It appears that under these conditions lysozyme does not seriously damage *E. coli*. If, however, lysozyme is injected into a suspension of cells in 0.5 M sucrose a few seconds before sudden dilution with 10 volumes of water, nearly complete lysis occurs and a highly viscous solution containing numerous free cell walls results. An electron micrograph of several of these cells walls is shown in figure 29, plate 3. Ultracentrifugal analysis of the viscous solution shows a pattern of sedimenting components typical of those observed in cell juices of *E. coli*. A sedimentation diagram is shown in figure 30, plate 4.

The influence of molecular size of the solute particle on the ability of *E. coli* to lyse was tested by suspending cells in various solutions isotonic with 0.5 M sucrose, adding lysozyme, and diluting the suspensions suddenly with 10 volumes of water. The degree of lysis was found to increase with increasing molecular weight of the solute particle. Thus, inorganic salts (NaCl, Na₂SO₄, C medium salts) and low-molecular-weight organic compounds (urea, glycerol, Tris, sodium succinate, sodium glutamate, casamino acids) caused no change in the suspension of cells. With glycine, however, lysis occurred slowly. Xylose, sorbitol, mannitol, inositol, and glucose did not allow lysis, but the marked streaming birefringence of the suspensions was much reduced, and observation of the bacteria in the dark-field microscope revealed that 50 to 100 per cent of the cells had assumed a spherical shape. Lactose, sucrose, maltose, and raffinose allowed complete and rapid lysis. On the other hand, when the osmotic pressure of a lysozyme-sucrose suspension of cells was slowly



Fig. 29. Electron micrograph of cell walls from *E. coli* treated with lysozyme and osmotically shocked. The walls appear as flattened disks which show a high incidence of double structure as though the original condition were of the bag-within-a-bag type. The dark rods are unbroken bacteria about 1.5 microns long.

lowered by the dropwise addition of distilled water, lysis failed to occur. Such "decompressed" cells were morphologically and metabolically indistinguishable from untreated bacteria.

From these results it was evident that the presence of lysozyme and a *considerable osmotic shock* were necessary to cause lysis. Since osmotic shock results from a relatively high intracellular concentration of solute, the opportunity was afforded to test the rate at which various solutes entered and left the cell. When *E. coli* were suspended in 0.5 M sucrose-lysozyme solution for varying lengths of time and suddenly diluted, complete lysis occurred only

after 30 to 60 seconds' equilibration of the cells with the suspending medium. Alternatively, when bacteria that had been equilibrated with a 0.5 M sucrose solution were first suddenly diluted and then lysozyme was added after increasing intervals, lysis occurred only in those cells that had less than 1 minute's exposure to the dilute medium. Thus, enough sucrose to cause osmotic lysis can enter or leave *E. coli* cells in about a minute.

Amino acid utilization by E. coli protoplasts. *E. coli* protoplasts may be efficiently prepared from exponentially growing cultures by suspending the cells in 0.5 M sucrose in C or Tris medium, adding lyso-

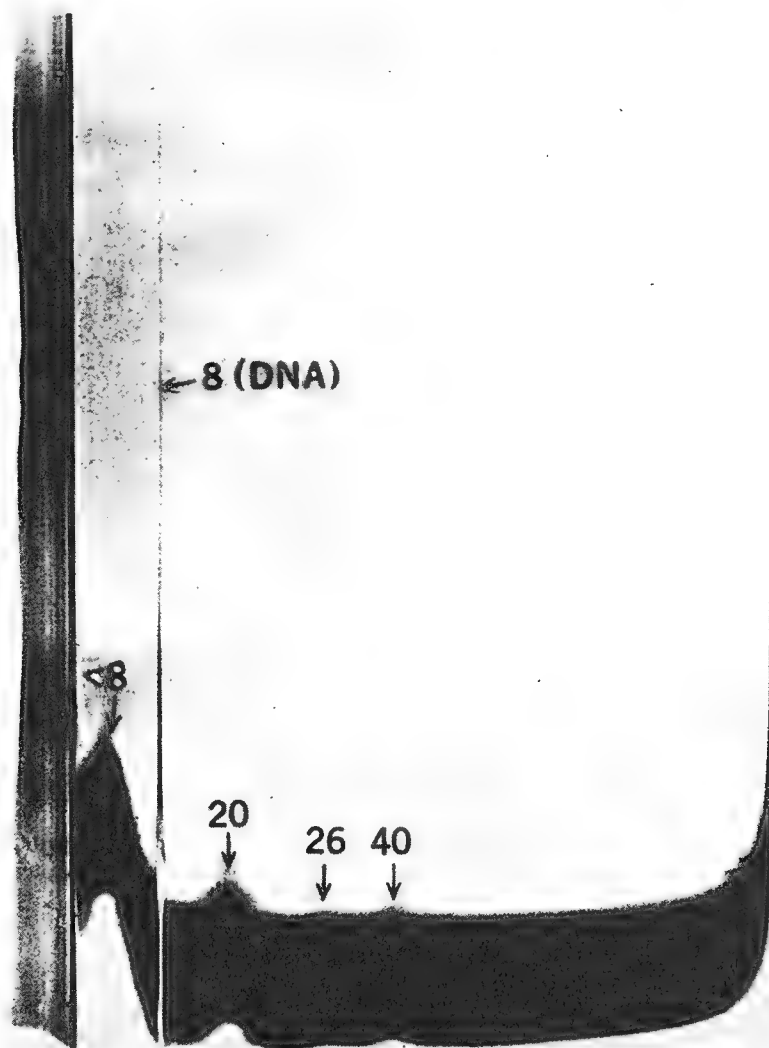


Fig. 30. Sedimentation diagram of *E. coli* extract prepared by lysozyme treatment and osmotic shock. The picture was taken about 30 minutes after the rotor reached a speed of 59,000 rpm. The most obvious components are the constituents labeled 40 S, 26 S, 20 S, and 8 S. The sharp spike is due to deoxynucleic acid. Such a simple pattern contrasts sharply with the exceedingly complex diagrams of the protein composition of *E. coli* as revealed by ion exchange (cf. fig. 36).

zyme (100 $\mu\text{g}/\text{ml}$), and suddenly diluting the suspension with an equal volume of water. In about 20 minutes at 25° C (10 min at 37° C) the suspension consists of 85 to 95 per cent protoplasts. Under these conditions protoplast formation requires considerable time and is a temperature-sensitive process. The walls of these forms enclose volumes from 2 to 50 times those of the rodlike forms. The protoplasmic body within the wall may occupy the

entire volume of the protoplast, may frequently be observed in a doubled condition within a single outer wall, or may adhere to one side of the wall, yielding a saucer-shaped inner structure. Since such protoplasts are morphologically altered *E. coli*, it was pertinent to inquire whether the structural changes would influence the maintenance and formation of amino acid pools and the synthesis of protein.

When growing *E. coli* were allowed to metabolize C^{14} proline in the presence or absence of lysozyme, no differences in the utilization of the amino acid could be detected. Equal C^{14} -proline pools were formed, and protein synthesis continued, as shown in figure 31. Lysozyme-treated

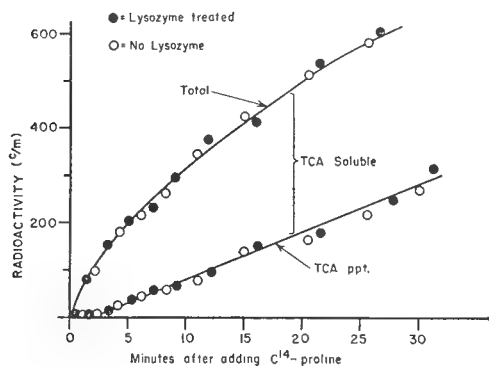


Fig. 31. C^{14} -proline utilization by cells growing in the presence of 100 $\mu\text{g}/\text{ml}$ lysozyme.

cultures grow indefinitely at the rate characteristic for *E. coli*. Thus, lysozyme treatment in itself had no apparent effect upon amino acid utilization or growth of *E. coli*.

Cells that had previously formed a C^{14} -proline pool and synthesized protein continued to maintain a large fraction of the original pool, increased the size of the pool, and continued to synthesize protein at a high rate even though they were undergoing the morphological change, rod-to-sphere, which followed lysozyme-sucrose osmotic-shock treatment. These findings are illustrated in figure 32.

The results of a more stringent test of the capacity of *E. coli* protoplasts to form an amino acid pool and to synthesize protein are shown in figure 33. For this experiment a protoplast suspension containing 96 per cent spherical forms was prepared and allowed to utilize C^{14} proline. It is evident that an amino acid pool is

formed and that protein is synthesized. It was also found that washing the protoplasts on membrane filters with isotonic media removed the labeled amino acid pool without causing lysis. Thus the amino acid pool is held so loosely that even mechanical mishandling of the protoplasts causes its loss.

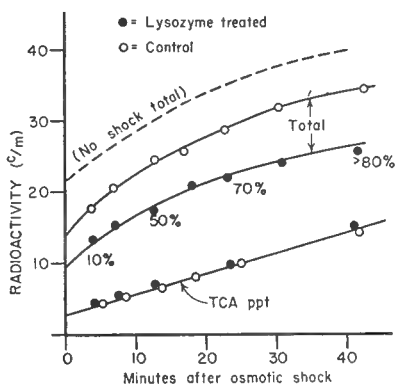


Fig. 32. C^{14} -proline utilization by *E. coli* after osmotic shock in the presence of lysozyme (solid circles). Percentages indicate the proportion of cells that were spherical as determined by direct observation in the phase-contrast microscope. Lysozyme was omitted in the control culture (open circles). The larger pool size for cells that received no shock or lysozyme treatment is shown by the dotted line.

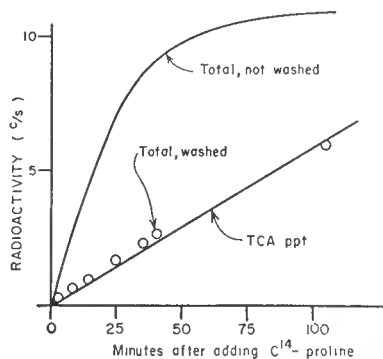


Fig. 33. C^{14} -proline utilization by a suspension of cells containing 96 per cent spherical forms (solid lines). Washing with isotonic medium removes the pool but does not lyse the cells (open circles).

Comment. This work was carried out in cooperation with Bill H. Hoyer and Edgar Ribi of the Rocky Mountain Laboratory, Hamilton, Montana, and with John Leahy, who was a Carnegie postdoctoral fellow. "Protoplasts" of *E. coli* are now only properly referred to as "spheroplasts" after the concordat of 1958 (S. Brenner et al., *Nature*, 181, 1713, 1958). McQuillen has written an informative chapter on bacterial protoplasts in *The Bacteria*, vol. 1, pp. 250-360, edited by I. C. Gunsalus and Roger Y. Stanier, Academic Press, New York, 1960. Ellis T. Bolton.

C. E. coli Nucleotide Pools

II.C.1 Phosphorus Incorporation

(Reprinted from Carnegie Institution of Washington Year Book 55, pp. 123-130, 1956.)

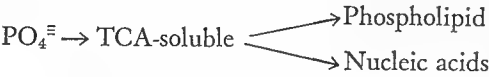
E. T. Bolton and R. B. Roberts

Phosphorus Incorporation

An extensive study of the flow of phosphorus in the metabolism of *E. coli* has been carried out during the past year. Phosphorus is a constituent of the nucleic acids, and phosphorus compounds such as adenosine triphosphate (ATP) have long been thought to be involved in the energy transfer mechanisms. Accordingly, it was considered possible that a detailed knowledge of the kinetics of phosphorus transfer might provide some clues either to the mechanisms of nucleic acid synthesis or to the way in which energy is supplied for protein synthesis.

ture of *E. coli*, and samples are taken to measure both the total incorporated and the TCA-insoluble fraction, the curve shown in figure 37 is obtained. (In all these experiments using P^{32} tracers, the usual phosphate buffer is replaced by tris-hydroxymethylaminomethane, "TRIS.")

The slow rise of the TCA-insoluble fraction suggests that it is derived from the soluble fraction according to the model:



According to this model, the radioactivity incorporated into the soluble fraction (Y)

TABLE 15
CHEMICAL FRACTIONATION OF *E. coli*

Fraction	P content (μmol)	Composition	Kinetics
TCA-soluble	200	$PO_4^{=}$, phosphorylated bases, etc.	Transient
Alcohol-soluble	150	Phospholipids	Stable
Insoluble	650	DNA, RNA	Stable

E. coli contains approximately 1000 micromoles of phosphorus per gram dry cells. In the growing cells the phosphorus is in three classes of compounds which are readily separated by simple extraction procedures, as shown in table 15.

Kinetics of the fractions. As simple extraction with TCA removes the transient intermediate compounds, the filter technique described above can be used to explore many of the general features of phosphorus metabolism.

When $P^{32}O_4^{=}$ is added to a growing cul-

ture per unit quantity of cells (*Q*) should be given by:

$$\frac{Y}{Q} = \frac{Y_o}{Q_o} (1 - e^{-bt})$$

where Y_o/Q_o is the steady-state value (200 μmol/gr) and *b* is a constant related to the rate of phosphorus incorporation. The observed curve is of this form, and the constant *b* can be determined.

Furthermore, when the cells are growing exponentially, $Q = Q_o e^{at}$, where the

constant a is given by the growth rate. Finally, the model predicts that the radioactivity incorporated into the insoluble fraction (Z) should be given by:

$$\frac{Z}{Q} = \frac{Z_0}{Q_0} \frac{1}{b-a} [b(1-e^{-at}) - a(1-e^{-bt})]$$

Figure 37 shows that this is the case, and the model gives a good approximation

in addition to the $19 \mu\text{mol/gr}/100 \text{ sec}$ incorporated. This exchange loss of phosphorus is somewhat variable, and sometimes runs as high as $20 \mu\text{mol/gr}/100 \text{ sec}$.

The stability of the TCA-insoluble fraction and the exchange loss from the soluble fraction can be shown in a more direct way by adding a large quantity of $\text{P}^{31}\text{O}_4^{3-}$, which dilutes the $\text{P}^{32}\text{O}_4^{3-}$ to such an extent

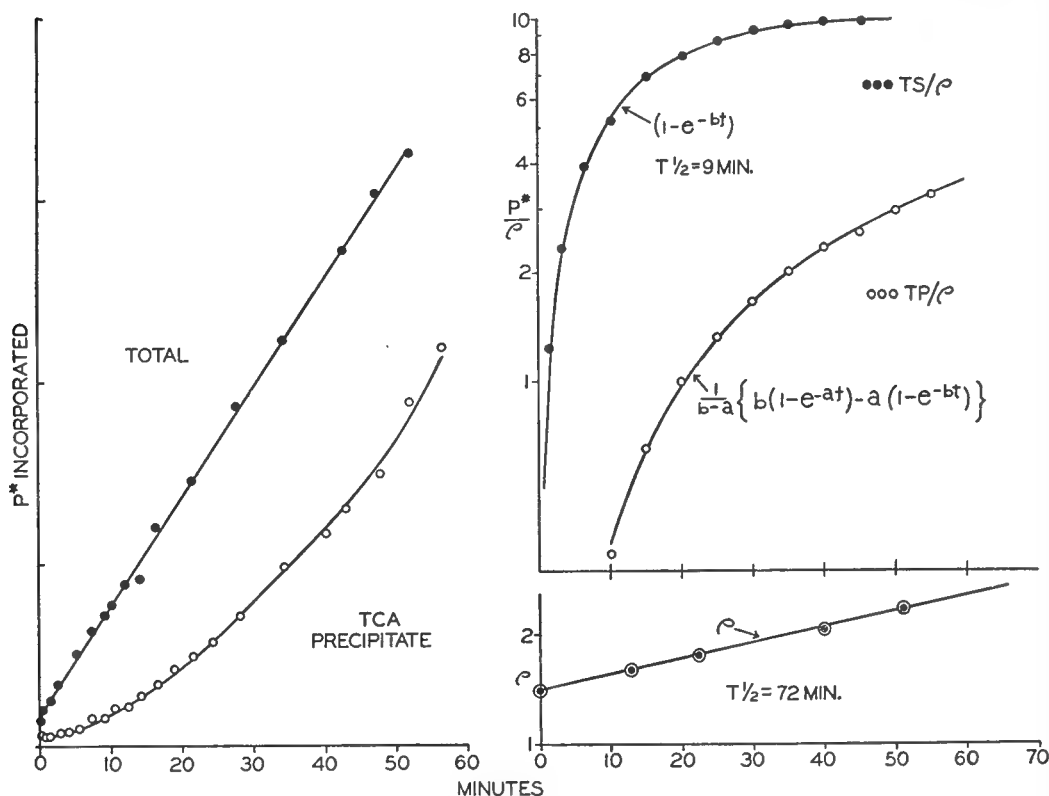


FIG. 37. The incorporation of $\text{P}^{32}\text{O}_4^{3-}$ by growing *E. coli*. The curves on the left are the experimental data. The same data are plotted in a different way for comparison with the curves calculated from the equations given in the text.

of the kinetics of phosphorus incorporation.

If the cells are growing in a steady state with a generation time of 60 minutes, they require a rate of incorporation of 1.9 per cent/100 sec or $19 \mu\text{mol/gr}/100 \text{ sec}$ to provide the phosphorus required for growth. The constant b evaluated above showed that the actual rate of incorporation is somewhat higher ($25 \mu\text{mol}$), which implies an exchange of $6 \mu\text{mol/gr}/100 \text{ sec}$

that its incorporation is reduced to a very low rate. As is shown in figure 38, after the $\text{P}^{31}\text{O}_4^{3-}$ is added the P^{32} content of the TCA-soluble fraction decreases exponentially, roughly one-half being lost to the medium and the other half being converted to nucleic acid and phospholipids.

It should be stressed that the equations above give only a good approximation to the kinetics of phosphorus metabolism. As will be shown below, the composition of

the TCA-soluble fraction is very complex, and only a portion contributes phosphorus to nucleic acid. These different compounds of the soluble fraction equilibrate among themselves rapidly so that extreme precision is necessary to show any deviations from the equations based on the simple model above. In addition, a part of the phosphorus of the soluble fraction is contained in kinetically stable molecules, but this fraction is so small that it does not introduce any deviation observable in an experiment of this type.

Conditions for phosphorus uptake. When glucose is omitted from the medium, no

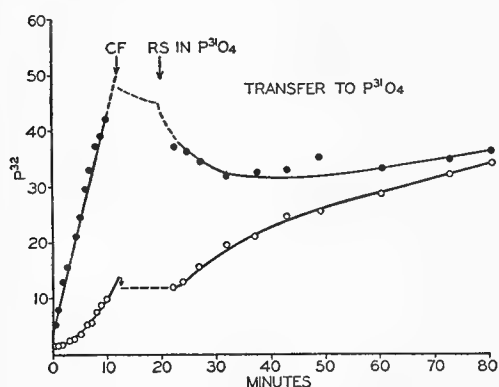


FIG. 38. Loss of P^{32} from the TCA-soluble fraction. Cells were grown in the presence of $P^{32}O_4^{3-}$, then transferred to a $P^{31}O_4^{3-}$ medium. The radioactivity of the TCA-soluble fraction is partly lost to the medium and partly converted to TCA-insoluble compounds.

phosphorus is incorporated. In a similar way, when the supply of glucose is exhausted the incorporation stops. These conditions are shown in figure 39. Neither lactate nor uridine added singly will replace glucose as an energy source, but a combination of the two will give some growth and phosphorus incorporation. This finding is in accord with other experiments which show that uridine accelerates adaptation to new carbon sources such as lactate, pyruvate, and acetate, perhaps by providing ribose in a usable form.

When nitrogen is omitted from the medium, or after the supply of nitrogen is

exhausted, there is no uptake of phosphorus. This is quite surprising, since the consumption of glucose continues at about one-half rate and potassium (which like phosphorus is involved in glycolysis) is exchanged. At first sight it might appear that phosphorus is needed only when nucleic acid is being synthesized. This is not the case in an adenine-requiring mutant of *Salmonella* (obtained from Dr. M. Demerec), which is closely related to *E. coli*. When nucleic acid synthesis is blocked by lack of nitrogen there is no uptake of phosphorus. In contrast, when nucleic acid synthesis is blocked by lack of adenine, phosphorus does enter the TCA-soluble fraction. It appears that the synthesis of some nitrogen-containing compound is necessary for the entry of phosphorus into the pool.

The addition of chloramphenicol at a concentration of 10 γ /ml, which reduces protein synthesis to one-twentieth its normal rate, has no immediate effect on phosphorus incorporation. Nor can any change in the phosphorus uptake be observed when amino acids are added. Evidently any connection between protein synthesis and phosphorus metabolism is too subtle to be easily observed by measurements of the kinetics of phosphorus transfer between the major fractions.

Competition. A number of compounds have been tested as competitors with PO_4^{3-} . Most of them, including adenosine triphosphate (ATP), glucose-1-phosphate, α -glycerol phosphate, etc., caused no reduction in the uptake of $P^{32}O_4^{3-}$. When glucose-6-phosphate, phosphoenol pyruvate, or fructose-6-phosphate (F6P) was added at concentrations equal to the concentration of phosphate (0.25 μ mol/ml), the uptake of PO_4^{3-} was markedly affected (fig. 40).

Two features of this curve require comment. The reduction does not occur immediately but only after a 5-minute lag, in marked contrast to other known competitive effects. The lag indicates that these compounds act indirectly, perhaps by raising the level of phosphate com-

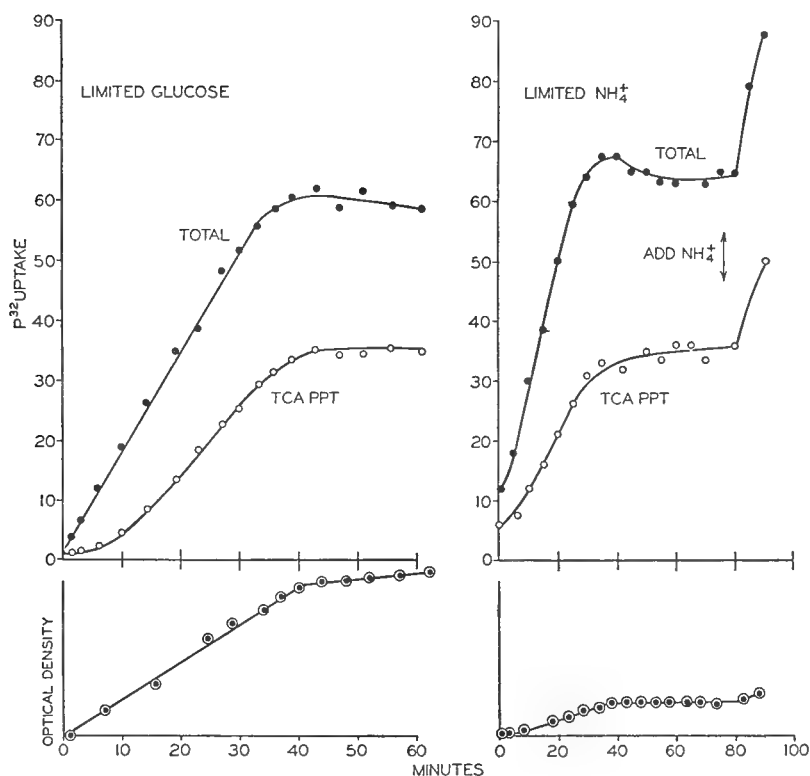


FIG. 39. The incorporation of phosphorus stops when the medium becomes depleted of glucose or NH_4^+ .

pounds within the cell. Secondly, the uptake of PO_4^{3-} is resumed at one-half the previous rate after a short period. Evidently the F6P is used up at a rate corresponding to the rate at which the cell uses glucose ($400 \mu\text{mol/gr}/100 \text{ sec}$). Most of its phosphorus returns to the medium as PO_4^{3-} and dilutes the $\text{P}^{32}\text{O}_4^{3-}$. The rate of

dephosphorylation is 10 times higher than the usual rate of phosphorus intake. This situation is surprising, because earlier experiments have shown that F6P supplies little carbon to the cell when glucose is also present. The findings are difficult to reconcile with the usual concepts of glycolysis.

Osmotic shock. The phosphorus compounds of the TCA-soluble pool exhibit a sensitivity to osmotic shock which is in some ways similar to, and in some ways different from, the osmotic sensitivity of the amino acid pool. To date, the experiments done with the phosphorus compounds have been carried out with cells grown in TRIS medium whereas the amino acid tests used C medium. No great difference would be expected between the two media, but the results are not strictly comparable until the tests have been repeated in the same medium.

Table 16 shows the results of a prelimi-

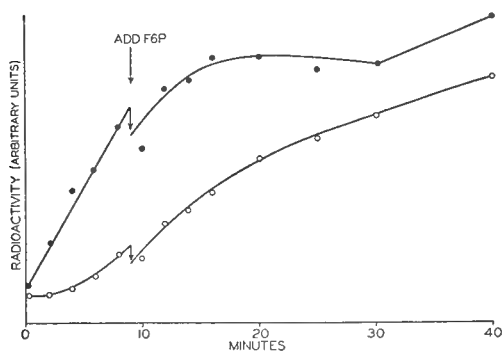


FIG. 40. The incorporation of phosphorus from $\text{P}^{32}\text{O}_4^{3-}$ stops 5 minutes after fructose-6-phosphate is added to the medium.

nary survey obtained by washing the cells on the filter with various fluids. As a large number of phosphorus compounds are found in the soluble pool, chromatograms were made of the material remaining after washing with several of the fluids. Phosphate appears to be the most readily extracted compound.

Phosphorus metabolism has another and different sensitivity to dilution. If a growing culture is diluted with water, the up-

may be due in part to differences in their localization within the cell. It is also possible that different-sized compounds might require different degrees of osmotic shock to be released from the same structure. In any event, the differences do demonstrate that the various components of the TCA-soluble pool are not all held within a common membrane that simply breaks on osmotic shock.

Composition of the soluble fraction. For further insight into the metabolism of

TABLE 16
REMOVAL OF TCA-SOLUBLE PHOSPHORUS BY WASHING

Washing fluid	Osmotic strength	Per cent removal
TRIS	0.31	0
	.15	35
	.04	55
	.01	75
Water	0	85
Glucose83	15
	.37	0
	.18	60
	.09	75
	.04	85
Sodium chloride31	0
Sodium acetate31	0
Sucrose31	0
Urea31	30
Glycerol31	80
Ethanol	0.31	85

Cells were grown with a limited glucose supply to give a constant pool of TCA-soluble phosphorus. They were removed from the medium by filtering, and washed while still on the filter with 2 ml of the listed fluids.

take of phosphorus into the cell as a whole stops abruptly for a period of several minutes and then is resumed at the original rate. Dilutions as low as 20 per cent, which remove very little phosphorus from the cell, will produce this effect. The treatment has no effect on the transfer of phosphate to the insoluble fraction, and is presumably connected with the mechanism involved in the entry of external phosphate.

The differences between the removal of phosphate and the removal of amino acids

TABLE 17
STEADY-STATE DISTRIBUTION OF PHOSPHORUS COMPOUNDS IN THE TCA-SOLUBLE POOL

Compound	Micromoles per gram dry weight
PO ₄ ⁼	80
ATP	20
ADP	12
GTP	7
GDP	7
UTP	15
UDP	20
CTP	5
CDP	5
10 unidentified spots.....	29
Total	200

phosphorus it is necessary to observe the individual components of the TCA-soluble fraction. For this purpose the cells are filtered and washed; the filter is promptly placed in a beaker containing 75 per cent ethanol. The TCA-soluble fraction and the phospholipids are extracted, leaving the nucleic acid still on the filter. The alcohol extract is then reduced in volume and applied to a sheet of Whatman No. 1 paper for two-dimensional chromatography.

Figure 41 shows a typical radioautograph of a chromatogram and indicates the spots that have been identified. The steady-state distribution is given in table 17. Most of these compounds are transient, and their quantity is affected by the differ-

ence between supply and demand. Hence, it is not surprising that the composition is somewhat variable, depending on the growth conditions. The guanine and cytosine compounds that are present in low concentration are subject to the greatest variations.

That some of these compounds are kinetically stable can be shown by growing cells in $P^{32}O_4^{3-}$ until a steady state is obtained and then replacing the $P^{32}O_4^{3-}$ by $P^{31}O_4^{3-}$. The transient compounds then decrease in radioactivity while the end products lose little of their radioactivity. This situation is shown in figure 42, where three minor components of the steady state are the most prominent. These compounds have not been identified, but they may well be coenzymes. The phospholipid also is a kinetically stable end product. The steady-state pattern of the pool has a remarkable degree of constancy. Cells that lack glucose might be expected to be deficient in the high-energy triphosphate compounds. They are not. Chromatograms of the soluble phosphorus compounds taken from cells which were incubated for 1 hour in media lacking either glucose, NH_4^+ , or PO_4^{3-} showed no significant change from the steady-state distribution. Even an adenine-requiring mutant showed no lack of adenine compounds after its growth was stopped by lack of adenine. This stability of the phosphorus system is in marked contrast to the instability of glutathione, which is completely used up when the cells lack sulfur.

Kinetics of the individual compounds. Some information about the source of nucleic acid phosphorus was obtained from studies of the kinetics of the individual phosphorus compounds of the soluble fraction. Cells were grown at 18° in a very low phosphorus medium ($0.01 \mu\text{mol/ml}$). $P^{32}O_4^{3-}$ was added to the culture, and after 1 minute's exposure all further uptake of P^{32} was stopped by diluting the culture medium with a large excess of $P^{31}O_4^{3-}$ ($50 \mu\text{mol/ml}$). Thus a pulse of radioactivity was taken into the cells, and its progress

could be traced (fig. 44). Samples were taken at intervals for chromatography and to measure the incorporation into nucleic acid. In a chromatogram of the earliest sample, taken after 12 seconds, only the PO_4^{3-} and the triphosphates of the nucleic acid bases show appreciable radioactivity (fig. 43). These compounds also showed a rapid decrease in radioactivity after the incorporation had stopped, then a secondary rise and decline (fig. 45). Radioactivity appeared more slowly in the diphosphates and reached a maximum later.

It seemed likely that the outermost phosphate of the triphosphate was responsible for the rapid rise and decrease whereas the secondary rise of the triphosphates was due to the appearance of radioactivity in the inner two phosphorus atoms. This hypothesis was tested and confirmed by degrading the ATP and uridine diphosphate (UDP) and measuring the radioactivity of each phosphorus atom separately. Figure 46 shows the radioactivity of the individual phosphorus atoms of ATP. The radioactivity appears first in the outer phosphorus, more slowly in the second phosphorus, and only after a considerable delay in the inner phosphorus.

These results give considerable insight into the circulating flows of phosphorus compounds. PO_4^{3-} and the four triphosphate compounds rise very quickly together, implying a rapid circulation that keeps them in equilibrium. The flow of glucose to pyruvate is 200 to $300 \mu\text{mol/gr/100 sec}$, and each mole of glucose should transfer 2 moles of phosphate to the triphosphates. Accordingly, the rapid equilibration of PO_4^{3-} and the external phosphorus of the triphosphate is not unexpected. This mechanism would not transfer any phosphorus to the interior positions.

The slow rise of the innermost phosphorus atom can also be explained. As the phosphorylated bases are incorporated into nucleic acid, newly formed ribosides must be phosphorylated. These reactions would

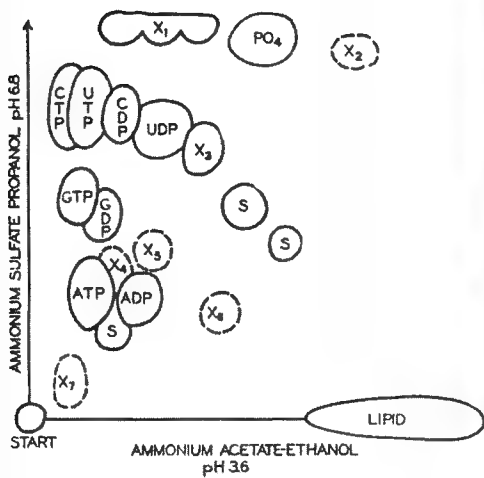


FIG. 41. Radioactive compounds which appear after paper chromatography of alcohol extracts of cells grown in the presence of $P^{32}O_4^{3-}$. The diagram shows the location of identified compounds. S indicates that the compound is a stable end product.



FIG. 42. Only a few compounds of the alcohol extract are kinetically stable and retain their radioactivity after an hour's growth in a medium containing $P^{31}O_4^{3-}$.

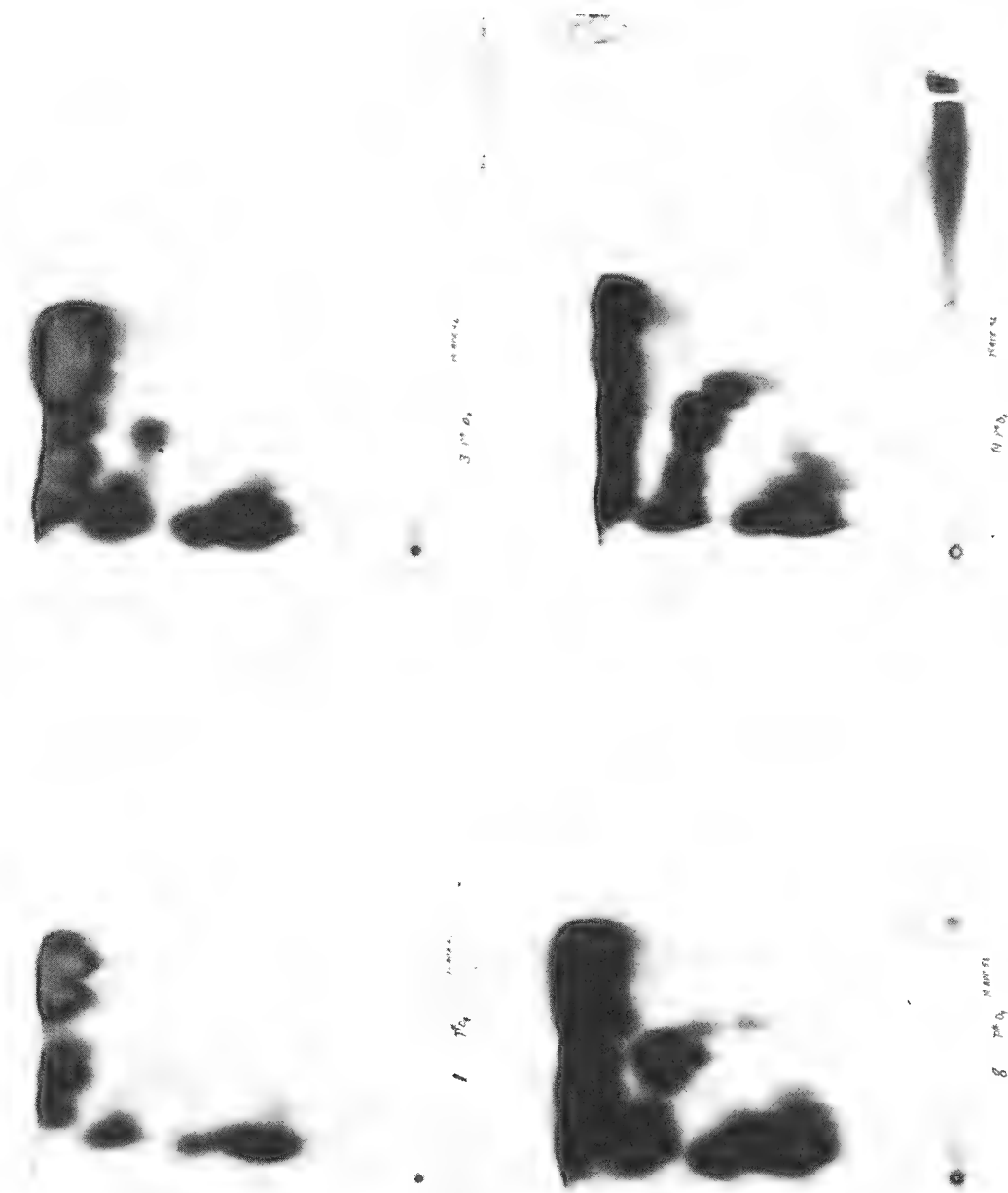


Fig. 43. This series of chromatograms shows how the radioactivity of a pulse of P^{32}O_4 appears first in the triphosphates and PO_4 and is transferred later to other compounds. Times: 12 seconds, 55 seconds, 3 minutes, and 14 minutes.

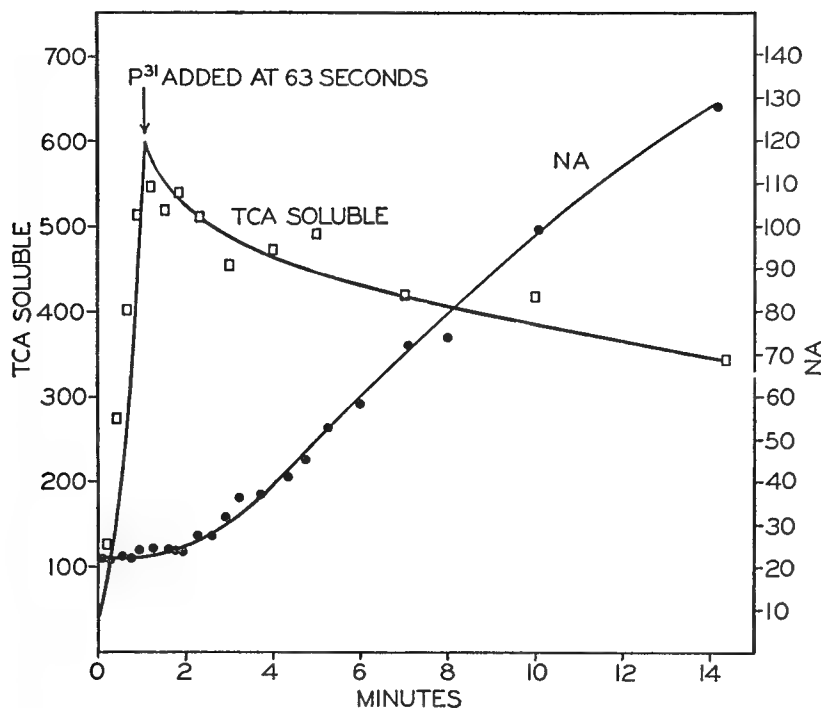


FIG. 44. Cells were exposed to high specific radioactivity $P^{32}O_4^{3-}$, and after 63 seconds $P^{31}O_4^{3-}$ was added; the radioactivity rapidly enters the TCA-soluble fraction and appears later in the nucleic acid fraction.

transfer phosphorus from the outer position to the inner positions at a relatively slow rate because the new ribosides are formed at roughly $15 \mu\text{mol/gr}/100 \text{ sec}$. Several minutes would be required to

bring the interior phosphorus to equilibrium by these reactions—roughly corresponding to the time interval observed.

Source of nucleic acid phosphorus. When the cells are growing in a steady state and the addition of the P^{32} causes no disturbance to the system, it can be assumed that the rate of incorporation of radioactivity into the nucleic acid will be proportional to the specific radioactivity of the nucleic acid precursors. Figure 43 shows that there is a very definite lag period in the incorporation of P^{32} into nucleic acid. This implies that a corresponding time elapses before the specific radioactivity of the nucleic acid precursors rises. Referring to figure 46, it can be seen that the nucleic acid phosphorus must be derived from the innermost phosphorus atom of the nucleotides, as the specific radioactivity of the other phosphorus atoms rises too rapidly. Further experiments are needed to determine whether one or all of the nucleotides contribute

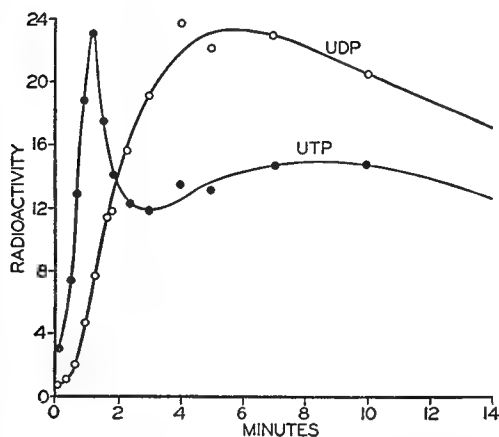
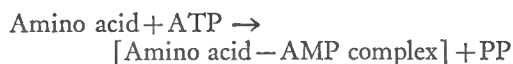


FIG. 45. The radioactivity of uridine-triphosphate and uridine-diphosphate was measured on the chromatograms. The other di- and triphosphate compounds showed similar kinetic behavior.

phosphorus to nucleic acid. This finding is consistent with the polymerization of diphosphate nucleotides observed in cell-free extracts by Grunberg-Manago and Ochoa.

Phosphorus metabolism and protein synthesis. No evidence for a direct relation between phosphorus metabolism and protein synthesis has been found. Chloramphenicol, which stops protein synthesis, has no effect on the incorporation of phosphorus. Cells in which the incorporation of amino acids is blocked by a lack of glu-

cose still contain plenty of nucleotide triphosphate which might be expected to supply the energy required for incorporation. No pyrophosphate is found in the cells. In spite of a prolonged search we have found no positive indication of the reaction:



which may be involved in the incorporation of amino acids.

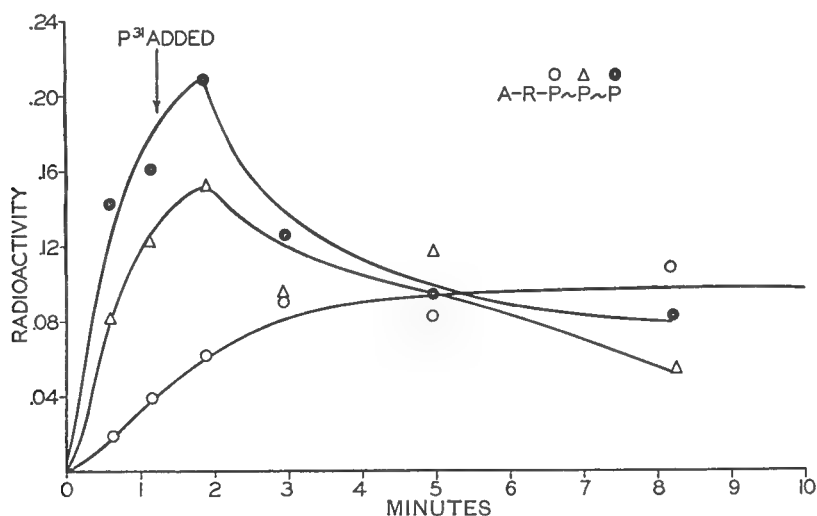


FIG. 46. Adenosine triphosphate was degraded to allow separate measurements of the radioactivity of the three phosphorus atoms. The outermost phosphorus atom is the first to pick up the radioactivity.

Comment. A few of the curves taken from this section appeared as examples of kinetic measurements in a lecture given by Roberts at Boulder, Colorado, in 1958 (*Rev. Mod. Phys.*, **31**, 170-176, 1959). The complete work was never published because of the unsatisfying conclusion stated in the last paragraph. The principal objective of this work was to demonstrate the rapid turnover of the central phosphorus atom of the nucleotides that would be expected from the amino acid activating reaction. For this purpose the results were equivocal.

Ellis T. Bolton and Richard B. Roberts.

II.C.2 The Synthesis of Ribosomes in *E. coli*, 1, The Incorporation of C¹⁴-Uracil into the Metabolic Pool and RNA

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ABSTRACT C¹⁴-uracil is rapidly incorporated by *E. coli* at low concentrations. Approximately half the radioactivity passes directly into RNA with very little delay. The remaining half enters a large metabolic pool and later is incorporated into RNA. The total rate of uptake (growing cells) is not greater than the requirement for uracil and cytosine for RNA synthesis. The size of the metabolic pool is not influenced measurably by the external uracil concentration. No evidence is found for the existence of a fraction of RNA which is rapidly synthesized and degraded.

A. INTRODUCTION

As a necessary part of the quantitative measurements of the time course of incorporation of C¹⁴-uracil into ribosomal RNA, reported in a succeeding paper (Paper III),¹ a study of the role of the metabolic pool has been carried out. The system has some novel features, for example, a large fraction of the C¹⁴-uracil is directly incorporated into RNA without measurable delay while the remainder is equilibrated with a rather large pool of phosphorylated compounds.

These observations are of interest not only with regard to the way exogenous uracil is handled by the cell but also have significant implications for studies of metabolic pools in general. Therefore they are described separately in this paper and their implications for the mechanisms of pool formation and maintenance are discussed.

B. METHODS

All of the experiments were carried out with *E. coli* ML 30 growing exponentially in C medium (Roberts *et al.*, 1955) using maltose as a carbon and energy source. The temperature was 37°C and the generation time about 51 minutes. 2-C¹⁴-uracil of specific activity

¹ For convenience, the many cross-references among this group of closely linked papers, published here together, will be written in this simplified style.

5 microcuries per micromole was obtained from the New England Nuclear Corporation. Membrane filters carrying thin samples of cells were counted in the tri-carb liquid scintillation counter (Paper III).

Measurements of total incorporation into cells and incorporation into TCA-precipitable RNA were carried out with the rapid filtering technique previously described (Britten, Roberts, and French, 1955). The ratio of radioactivities entering cytidylic and uridylic acids of RNA was determined by paper chromatography with the following solvent: tert-butyl alcohol, 12 N HCl, H₂O 70/6.7/23.3. For this purpose samples of whole cells washed in 5 per cent TCA were hydrolyzed overnight at 37°C in 0.380 N KOH. The KOH was then adsorbed on fine-grain carboxylic ion exchange resin and the supernatant placed directly on the chromatogram. Only insignificant amounts of radioactivity were observed except in the uridylic and cytidylic regions on the chromatogram.

C. RESULTS

1. *Major Features of C¹⁴-Uracil Incorporation.* The results of an experiment in which 10^{-7} M C¹⁴-uracil was supplied to cells are shown in Fig. 1. The uracil

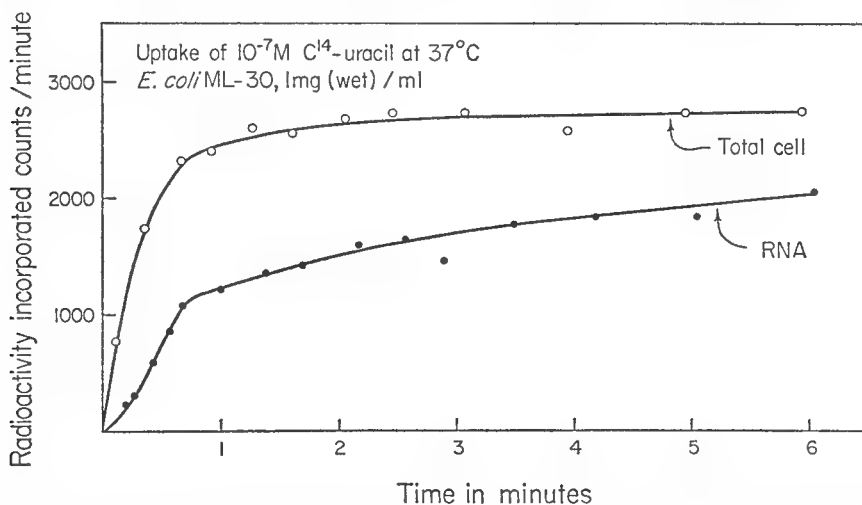


FIGURE 1 Incorporation of C¹⁴-uracil into the metabolic pool and the RNA of *E. coli*. The difference between the two curves is the radioactivity in the metabolic pool. Initial uracil concentration was 10^{-7} M.

is almost completely removed from the medium by 40 seconds. During this period 2-C¹⁴-uracil enters the RNA at a rate corresponding to about one-third of the total rate of uptake by the cells.

After 40 seconds when the C¹⁴-uracil is completely removed from the medium, the rate of incorporation into RNA suddenly falls by a factor of about 5. During this second phase the rate of incorporation of uracil label falls slowly.

A semi-log plot made of the radioactivity of the pool as a function of time during this second phase shows an exponential decay with a time constant (decay to $1/e$) of about 9.0 minutes.

Fig. 2 shows the qualitatively similar results of an experiment at 10^{-6} M. The same two phases in the incorporation into RNA are observed. However in this case, the first phase, during which the C^{14} -uracil is still present in the medium, lasts for 2 to 3 minutes. The transition to the second phase is not quite so abrupt, but again an exponential decay of the pool radioactivity with a time constant of about 10 minutes is observed.

The basic properties of this system become clear when experiments at higher

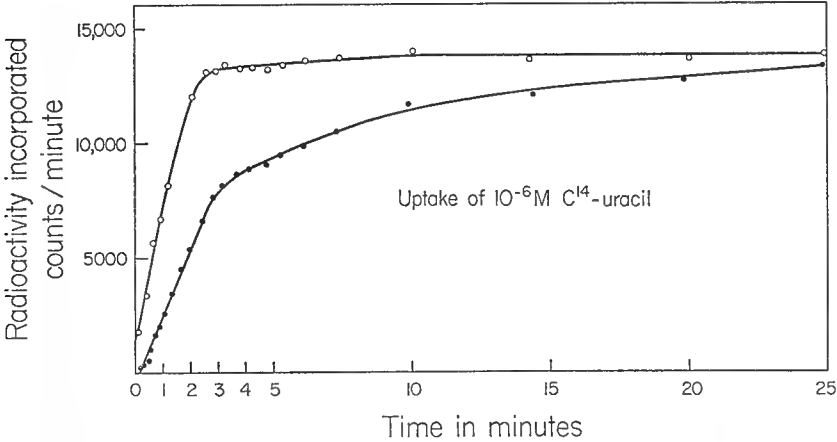
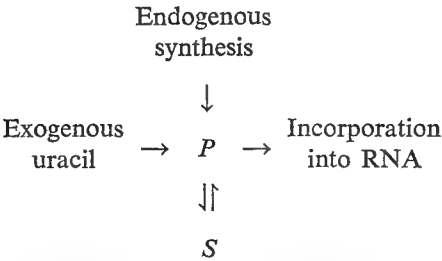


FIGURE 2 Incorporation of C^{14} -uracil into the metabolic pool and RNA of *E. coli* ML 30 at 37°C . Initial uracil concentration, 10^{-6} M. Cell concentration, 0.59 mg (wet) per ml. Open circles, total incorporation. Solid circles, incorporation into RNA.

concentrations are examined. Fig. 3 shows the incorporation of 2- C^{14} -uracil into RNA at 5×10^{-5} M and 10^{-3} M. In these cases the amount taken up into the pool is such a small fraction of the total uracil present that its determination by the difference between total and RNA is subject to great uncertainty and the data are not presented. However, the incorporation into RNA supplies the necessary information. Two phases in the curves are again observed; however, in these cases the rate of incorporation into RNA at later times is just greater than twice the initial rate.

A simple interpretation of these experiments is indicated in the following diagram:



This schematic diagram is presented here to define the symbols P and S in order to simplify the language used in later sections. P represents a very small pool or se-

quence of reaction steps leading from uracil to a chemical form suitable for incorporation into RNA. *S* represents a large pool of compounds which can exchange with some uracil compound in *P*. The rate of exchange between *S* and *P* is not fast, and equilibrium between the specific radioactivity of *P* and *S* requires several minutes, at least. *P* then effectively forms a bypass, around the large pool, for the entry of exogenous uracil into RNA.

2. *Lack of Expansion of the Pools.* The question must be raised as to whether the amounts of compounds in the states represented by *S* and *P* are

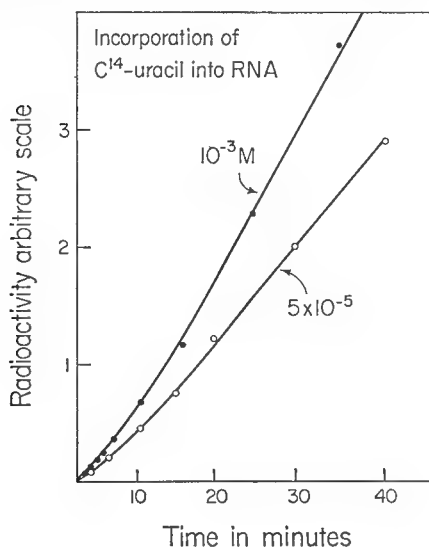


FIGURE 3 Incorporation of C^{14} -uracil into the RNA of *E. coli* ML 30 at 37°C . Solid circles, initial concentration 10^{-3} M. Open circles, initial concentration 5×10^{-5} M. The ordinate scales for the two curves are not related. The abscissa scale is proportional to increase in cell mass, with sample times indicated.

dependent on the concentration of exogenous uracil. That is, are these two pools expandable? This question is answered by an experiment (Fig. 4) in which C^{12} -uracil (4×10^{-5} M) was supplied to the cells for 10 minutes before the C^{14} -uracil was added. An identical curve within very small limits of error (< 5 per cent) was obtained in a simultaneously carried out control experiment in which an indential quantity of C^{14} -uracil was added with the C^{12} -uracil at zero time. The concentration (4×10^{-5} M) is sufficient to achieve the maximum rate of entry of uracil into the cells. Therefore if the size of the two pools depended on the external concentration, a significant difference should appear between the control and the experiment in which C^{12} -uracil was added beforehand.

If *S* were expanded its specific radioactivity should rise rapidly in the control since the new material flowing in would be radioactive. On the other hand in the experiment in which the C^{12} -uracil was added beforehand the specific radioactivity should rise more slowly since a large amount of unlabeled uracil compounds would have been present in the pool at the time the C^{14} -uracil was supplied. It is evident that the pool *S* was not expanded by a measurable amount—certainly not by more than a small fraction of its preexisting size.

If any appreciable amount of unlabeled uracil compounds exists in state *P*, a delay in the entry of the radioactivity from exogenous uracil into RNA should be observed. There is no evidence of such a delay in the experiment of Fig. 4 after correction is made for the curvature due to the rising contribution of radioactivity from *S*. Without correction for this curvature the data for the first 5 minutes appear to extrapolate to a delay time of 10 seconds. The precision of the data and correction are such that the maximum actual delay time along the route through *P* must be less than 5 seconds.

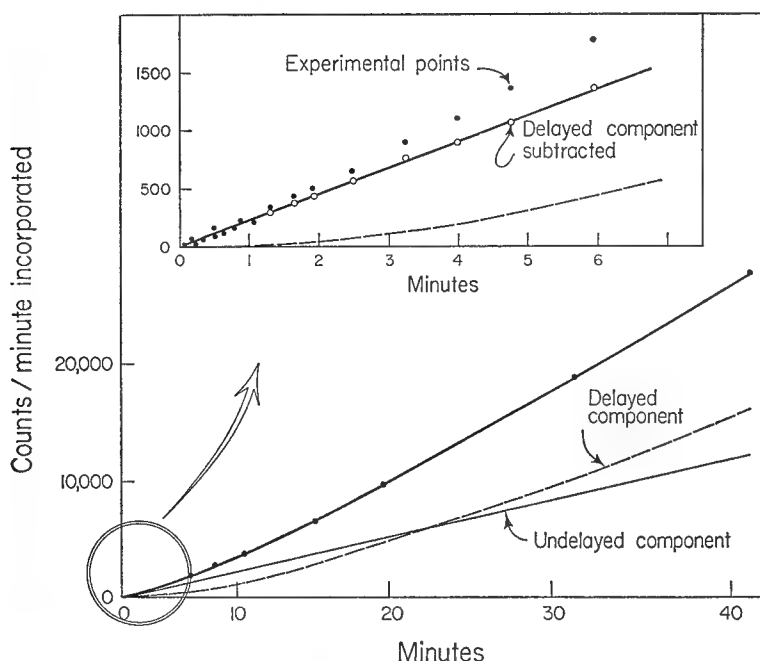


FIGURE 4 Incorporation of C^{14} -uracil into the RNA of *E. coli* ML 30 after 10 minutes' pretreatment with C^{12} -uracil at 4×10^{-6} M. The upper figure presents the data at early times with expanded scales. The light, solid, and dashed curves represent the best separation of the curve into a linear component and a component delayed by the pool (*S*). Solid circles are experimental values. For the open circles the component delayed by *S* was subtracted. The abscissa scale is proportional to increase in cell mass, with sample times indicated.

It can be concluded that the amount of compounds in the states represented by *P* is so small that it cannot be adequately measured by these techniques, and that it remains small in the presence of moderately high concentrations of uracil.

3. *Conversion of Uracil to Cytosine.* Previous experiments (Roberts *et al.*, 1955) have indicated that uracil is converted to a great extent to cytosine compounds by *E. coli*. The questions that arise now are: what is the rate of this process, and to what extent is the second phase in the incorporation into RNA influenced by this conversion?

TABLE I
CONVERSION OF URACIL COMPOUNDS TO CYTOSINE COMPOUNDS

Culture density	Sample A		Sample B	
	Time	C*/U*	Time	C*/U*
<i>mg wet/ml</i>				
2.8	16''	0.075	15'-00''	0.614
1.4	31''	0.100	15'-30''	0.618
0.70	1'-00''	0.184	16'-00''	0.605
0.35	2'-4''	0.230	17'-34''	0.650
0.175	4'-03''	0.280	19'-00''	0.625
0.087	8'-00''	0.392	23'-00''	0.675
0.044	16'-22''	0.570	31'-00''	0.663

1.1×10^{-7} M C^{14} -uracil was added to seven cultures at the cell densities listed in column 1. An aliquot of the culture was brought to 5 per cent TCA at the time listed in column 2. TCA was added to the remainder of the culture at the time listed in column 4. The ratio of radioactivity of cytidylic acid and uridylic acid was determined after washing, alkaline hydrolysis, and chromatography.

In the first experiment to be described, 2- C^{14} -uracil was supplied to growing cultures at a concentration of 1.1×10^{-7} M. In order to vary the period of time during which incorporation into RNA occurred and still achieve efficient utilization of tracer at early times, seven cultures were set up simultaneously at different cell densities. The cell densities were chosen so that the C^{14} -uracil in the medium would be nearly exhausted at the time sample A was taken, as shown in Table I.

As a control a part of each culture (B) was incubated an additional 15 minutes

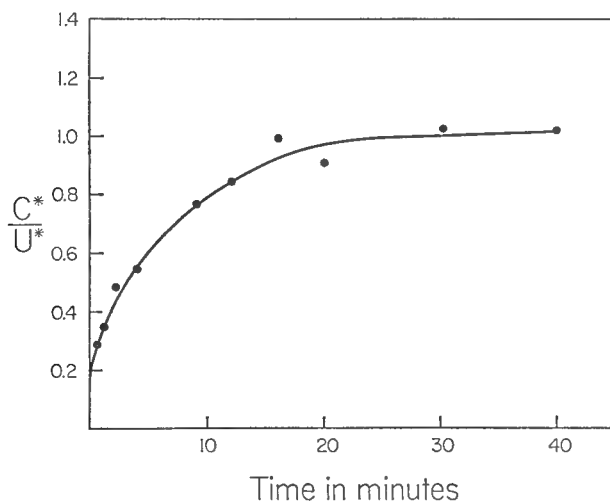


FIGURE 5 The ratio of the radioactivity of cytidylic acid to that of uridylic acid derived from the RNA of growing cells supplied 2×10^{-6} M C^{14} -uracil at zero time. Determined from chromatography after alkaline hydrolysis of samples taken into 5 per cent TCA.

TABLE II
RELATIVE RATES OF ENTRY OF C¹⁴-URACIL
INTO THE CYTIDYLIC AND URIDYLIC ACIDS OF THE RNA
Uracil concentration 2×10^{-5} M

	First phase 0 to 2 min.	Second phase 20 to 40 min.
Total	43	100
Uridylic	31	50
Cytidylic	12	50

so that almost all of the radioactivity which had entered *S* would be swept on into RNA. The ratio of radioactivity in cytidylic acid to that of uridylic acid in the RNA is almost constant in these samples. The slight increase is probably due to the fact that 15 minutes was not quite long enough to sweep all of the radioactive compounds out of *S*. This result is to be expected when there is not an induced (*i.e.* time-increasing) rate of conversion of uracil to cytosine, and implies that such an effect does not occur at this concentration of exogenous uracil.

The results shown in column 3 (Table I) indicate that uracil compounds are rather slowly converted to cytosine compounds and that after conversion the labeled cytosine compounds mix with a large endogenous pool of cytosine compounds before entering the RNA. The fact that some labeled cytidylic acid is observed in the RNA at the earliest time point suggests that there may be a small bypass around the pool of cytosine compounds.

On the basis of this experiment an estimate can be made of the kinetics of appearance of radioactivity separately in uridylic acid and cytidylic acid of the RNA. In the experiment at nearly the same concentration shown in Fig. 1, 40 per cent of the radioactivity was incorporated during the first phase. At the end of the first phase (at 40 seconds) the C*/U* ratio would be about 0.15 and at the end of the second phase about 0.66. Taking the total radioactivity incorporated into RNA as 100 per cent, we may then say: of the 40 per cent incorporated in the first phase, 35 per cent appeared in uridylic acid and 5 per cent in cytidylic; of the 60 per cent incorporated in the second phase 25 per cent appeared in uridylic and 35 per cent in cytidylic.

On Fig. 5 are shown the results of an experiment in which 2×10^{-5} M C¹⁴-uracil was supplied to a growing culture of cells. Small samples were added to an equal volume of 10 per cent TCA, filtered, and assayed for radioactivity in order to measure the total incorporation. At several times larger samples were precipitated with TCA and the ratio of the radioactivity of the cytidylic and uridylic acids of the RNA was determined. The shape of the total incorporation curve was essentially identical to those shown in Figs. 3 and 4 and is therefore not reproduced.

The ratio of the radioactivity of the cytidylic to the uridylic acids of the RNA

is higher throughout the time course of this experiment than it was in the experiment at 1.1×10^{-7} M. Thus there is apparently a dependence of the conversion rate on the external concentration of uracil. This is not inconsistent with the lack of expansion of the pools. If the pools are in fact organized in the cell or under tight internal control, a considerable shift in chemical equilibria could occur without a measurable change in pool size.

From the data on Figs. 4 and 5 the rates of incorporation of radioactivity from uracil into the uridylic and cytidylic acids of the RNA may be calculated during the two phases. The results are given in arbitrary units in Table II.

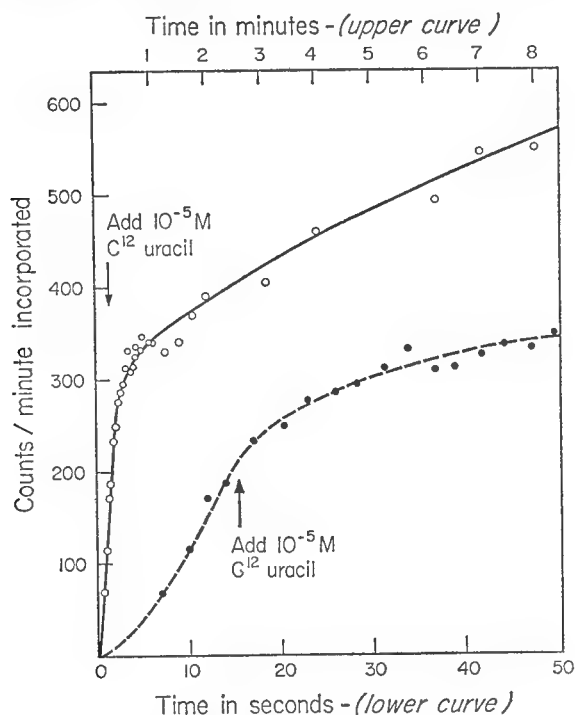


FIGURE 6 Incorporation of C^{14} -uracil into RNA in a chase experiment. 10^{-7} M C^{14} -uracil was added initially. At 15 seconds 10^{-5} M C^{12} -uracil was added. The lower curve (solid circles, dashed line) is identical to the upper curve but with an expanded time scale.

From these data it appears that there is an increase in the rate of incorporation into uridylic acid in the second phase. Moreover both experiments show that it is not purely the conversion of uracil compounds to cytosine compounds and delay by the pool of cytosine compounds which give rise to the second phase in the incorporation curves. This result is consistent with the existence of pools of UDP and UTP as well as CDP and CTP (Roberts, 1956). It also appears that there is some rapid conversion of uracil compounds to cytosine compounds since C^*/U^* ratio is already about 0.3 in the earliest point on Fig. 5. Therefore when C^{14} -uracil is supplied,

uracil and cytosine compounds become labeled both in states *P* and *S*, and conversion of uracil to cytosine may occur in both states.

4. *A "Chase" Experiment.* The word "chase" is used to represent a situation in which a labeled metabolite is present for a short period of incorporation and then followed by the incorporation of unlabeled metabolite.

Fig. 6 is an example of such a chase experiment. The cell density (1.7 mg wet/ml) is at the upper safe limit for steady exponential growth with good aeration for these cells. The initial C^{14} -uracil concentration (10^{-7} M) was the same as that of the experiment of Fig. 1. At this cell density, therefore, the first phase would be expected to last for about 25 seconds without a chase. The ratio of rates of incorporation of radioactivity into RNA before and after the chase was about 30 to 1.

The rate of incorporation after the chase is due to the quantity of radioactive compound which has entered the large pool, *S*. The specific activity of *S* is only slowly reduced by dilution after the chase. The relative entry of radioactivity into *S* can be minimized only by reducing the period of exposure to the tracer. Therefore with this system high ratios of the rate of incorporation into RNA before and after chase are achieved only with such short pulse times.

Such a short period of rapid incorporation followed by a sudden fall in the rate of incorporation is a favorable circumstance for the recognition of an RNA fraction which is rapidly synthesized and degraded. Such a fraction would be expected to fall rapidly in specific radioactivity very shortly after the chase. No fall in the TCA-precipitable RNA radioactivity is observed.

D. DISCUSSION

1. *The Pool of Uracil Compounds.* A striking result of these experiments is that external uracil is incorporated rapidly into RNA without delay even though there exists a large pool of uracil compounds which can also be incorporated into RNA. The implications of this result are represented on the schematic diagram. The meaning of the symbols *S* and *P* has been previously defined. This diagram not only suggests a possible mechanism for the bypass around the pool but also explains the detailed shape of the incorporation curves at high and low concentrations. The specific radioactivity of *P*, and therefore the rate of incorporation of radioactivity into RNA is the net result of the mixing of labeled uracil entering from outside with unlabeled uracil compounds internally synthesized and with uracil compounds from *S* which may be more or less labeled depending on the previous 15 minutes' history.

In the experiments of Figs. 1 and 2, as long as C^{14} -uracil enters the cell, radioactivity is incorporated into RNA at a relatively rapid rate. According to the diagram this is to be expected as long as exogenous uracil enters *P*. During this time radioactivity also enters *S*. When the C^{14} -uracil in the medium is exhausted the rate of incorporation abruptly falls to a new rate. Radioactive uracil compounds continue to flow from *S* into *P* and thence into RNA. The specific radioactivity of these com-

pounds and thus the rate of incorporation of radioactivity into RNA during the second phase will be determined by the amount of radioactivity that has entered *S* during the first phase. The relative rate of incorporation of radioactivity into RNA in the second phase is considerably higher in Fig. 2 than in Fig. 1. This is simply the result of the longer period during which radioactive uracil compounds have entered *S* yielding a higher specific radioactivity at the end of the first phase.

During the second phase the rate of entry of radioactivity slowly falls. In a number of experiments, the amount of radioactivity in the pool during the second phase (the difference between the total radioactivity in the cell and that in the RNA) was plotted on semilog paper. In every case a good straight line resulted and the time constant (decay to $1/e$) varied between 9 and 12 minutes. This is consistent with the diagram since the specific radioactivity of *S* should slowly fall as it is diluted by the flow of internally synthesized compounds.

In the experiments at higher concentrations (Fig. 3) the exogenous uracil lasts sufficiently long for the specific radioactivity of the uracil compounds of *S* to approach that of the uracil supplied. As a result the rate of entry of radioactivity into RNA slowly rises to its final value over a period of 10 to 20 minutes.

The ratio of the rate of flow into RNA to that into *S* is about the same in all of these experiments. However, the absolute rate of entry of uracil into the cell does increase with concentration, and reaches a saturation value not far different from the requirement for uracil plus cytosine for RNA synthesis. From a number of experiments the concentration at which the rate reaches half the saturation value appears to be about 2×10^{-7} M.

The quantity of compounds in the state *S* cannot be precisely evaluated from these experiments, due to the conversion of uracil compounds to cytosine compounds and to the existence of pools of both classes of compounds. Since the time constant of *S* is about 10 minutes and the flow through it just greater than half of the total flow into RNA, the total quantity of uracil and cytosine compounds can be roughly estimated to be the amount (of both classes) that is utilized for RNA synthesis in 5 to 8 minutes.

The best estimate of the quantity of uracil compounds in state *P* comes from the chase experiment (Fig. 6). The incorporation of C^{14} -uracil into RNA neither starts entirely abruptly nor drops to the second phase rate abruptly. The initial delay appears to be between 3 and 6 seconds and the time constant for the fall to the second phase rate appears to be about 7 seconds. The upper limit on the amount of uracil compounds in state *P* is therefore about the amount required for 5 seconds of total RNA synthesis or 0.1 per cent of the total uracil in the RNA. This must be considered an upper limit since there may be some delay in the entry of uracil into the cell.

The scheme proposed here to explain the observations on the incorporation of C^{14} -uracil has striking similarities to the "carrier model" (Britten and McClure,

1962) which has been proposed for the proline pools of *E. coli*. The experimental facts in the two cases differ in almost every conceivable way. The proline pool existing in the absence of supplement is very small; the maximum rate of uptake is 10 times the requirement for protein and the pool can be expanded to a very large size. There is no evidence of a bypass around the pool for entry of external proline into proteins. The only similarities are: both compounds are effectively taken up at low external concentrations; internal synthesis is effectively shut down at moderate concentrations; both compounds are incorporated into macromolecules.

However, it appears that by simply changing the values of a few of the reaction rate constants the predictions of the carrier model will be altered to agree with the observations presented in this paper on the incorporation of uracil. The reactions postulated in the carrier model are:



U symbolizes uracil compounds, ignoring all of the important chemical changes which must be carried out in a set of subsidiary reactions. E is the carrier, which is mobile within the cell and specific for the class of uracil compounds. R represents a set of specific storage sites. \overline{UX} is the final reaction complex in the internal synthesis of uracil compounds. Uracil compounds are freed from it only through the reaction with free carrier, E . Thus equation (4) symbolizes the mechanism for control of the rate of internal synthesis. The reduction of the concentration of free carrier due to reaction with an excess of uracil will effectively shut down internal synthesis.

\overline{UE} is the complex between the carrier and uracil compounds, and is equivalent to P in the schematic diagram. \overline{UR} is the complex of uracil compounds with storage sites and is equivalent to S in the schematic diagram. If the equilibrium for reaction (2) is far to the right, then there will be little free R in any circumstance and a constant non-expandable pool (S) will result. If, in addition, the rates in reaction (2) are not fast, the observed slow equilibrium in specific radioactivity between S (\overline{UR}) and P (\overline{UE}) will result. If the dissociation constant in reaction (1) is small, the amount of free E will always be small and the rates in reaction (2) will hardly depend on the external uracil concentration, as observed. Further, the loss of uracil compounds will be small.

Reaction (1) represents the uptake of uracil into the reaction system. It is presumed that uracil can reach the carrier at a sufficient rate. Since the concentration of free E is probably small, it must have a high affinity for uracil.

Qualitatively, at least, the set of reactions (1), (2), (3), and (4) is consistent with the observations on the incorporation of uracil into the cell and into RNA.

These equations have been derived from the carrier model. However, they are sufficiently general that they may well be consistent with other models utilizing entirely different mechanisms. The ability of these equations to express compactly the characteristics of two such apparently different systems (uracil and proline) is both surprising and rewarding. It is suggestive that there is a kernel of truth in them.

2. *Absence of Evidence for Degradation of RNA.* The messenger hypothesis of Jacob and Monod (1961) proposes the existence of a small fraction of RNA which is synthesized, used as template for protein synthesis, and degraded. A rapidly labeled fraction of the RNA of bacterial cells has been observed (Bolton and Britten, 1958; Roberts, 1958; Britten *et al.*, 1959; Aronson *et al.*, 1960; Gros *et al.*, 1961). However, the rate of incorporation of labeled precursors into this fraction is exactly that which would be expected if it were a compulsory precursor to ribosomal RNA (Paper III). Hence its rapid labeling provides no proof of its degradation. Evidence for degradation must be obtained directly.

The delay in the labeling of ribosomal RNA shows that more than 90 per cent of the flow into ribosomes must pass through this fraction (Paper III). Hence, if it is not a compulsory macromolecular precursor to ribosomal RNA, the degradation products must be effectively reutilized. Consequently, if the synthesis and degradation can be represented as an exchange with an intermediate on the pathway for ribosomal RNA synthesis, the rate of exchange must be at least 10 times the flow to ribosomal RNA. The average lifetime of molecules in the fraction would therefore be less than 15 seconds, which seems too short for template material in view of the $2\frac{1}{2}$ minute lifetime of the enzyme-forming unit for β -galactosidase (Pardee and Prestidge, 1961; Boezi and Cowie, 1961).

Further, if the hypothetical degradation products could mix with the metabolic pool of nucleotide precursors, then a fall in radioactivity of the RNA should be observed in the experiment shown in Fig. 6. No fall in radioactivity is observed after the chase, although at 15 seconds all of the RNA radioactivity must be present in the rapidly labeled fraction. Clearly, if any significant fraction of the degradation products entered *S* there would be an observable fall in RNA radioactivity since the product of specific radioactivity and rate of utilization of compounds from *S* is measured by the slow rate of incorporation into RNA after the chase. Equally, if the degradation products entered *P*, a certain fraction (probably more than half) would pass into *S*, since the kinetic experiments require that there be a significant circulation between the compounds of *P* and *S*. In this case, therefore, there should also be a fall in the RNA radioactivity.

Thus these experiments show no indication of degradation of the rapidly labeled fraction of RNA. Degradation could have escaped detection if only a small fraction of the newly made RNA were degraded or if the degradation proceeded only to the level of oligonucleotides. In this case the ribosomal RNA would be assembled almost completely from the "second hand" oligonucleotides. A third alternative is

that the proposed messenger is distinct from the rapidly labeled fraction. A small fraction of RNA which utilized precursors only from a slowly labeled pool such as *S* would escape detection in these experiments.

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II.C.3 Incorporation of Ribonucleic Acid Bases into the Metabolic Pool and RNA of *E. coli*

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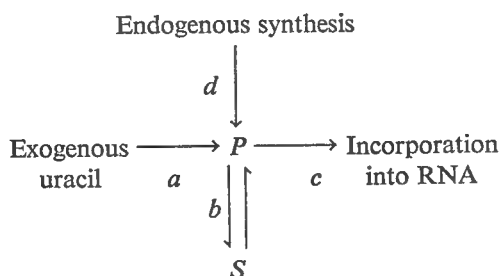
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ABSTRACT Labeled cytosine, adenine, and guanine are rapidly incorporated by *E. coli*. A fraction of the radioactivity passes directly into RNA with very little delay. The remainder enters a pool before being incorporated into RNA. The fractions entering the pool and the time constants for equilibration of the specific activity of the pool are widely different for the four RNA bases.

INTRODUCTION

Studies of the incorporation of C¹⁴-uracil (McCarthy and Britten, 1962) showed direct entry of radioactivity into the ribonucleic acid (RNA) without delay by the large pool of nucleotides. The following schematic diagram was proposed to describe the kinetics of uracil incorporation.



P represents a very small pool or sequence of reaction steps leading from uracil to a chemical form suitable for incorporation into RNA. *S* represents a large pool of compounds which can exchange with some uracil compound in *P*. The rate of exchange between *S* and *P* is not fast, and equilibrium between the specific radioactivity of *P* and *S* requires several minutes, at least. Thus, according to this model, external uracil tracer may enter RNA without being delayed by passage through

the large pool (*S*). *P* then effectively forms a bypass around the large pool for the entry of exogenous uracil into RNA.

Since an alternative explanation of the undelayed entry of radioactivity into RNA has been proposed (Gros *et al.*, 1961) it seemed worthwhile to examine the kinetics of incorporation of the three other RNA bases. The additional information reported here clarifies the situation. The qualitative similarities and the quantitative differences in the kinetics of incorporation of the four RNA bases amply demonstrate the existence of direct pathways into RNA which bypass the large pools.

METHODS

All experiments were carried out with *E. coli* ML 30 growing exponentially in C medium (Roberts *et al.*, 1955) at 37°C with maltose as carbon and energy source. The generation time was 51 ± 2 minutes. The radioactive tracers used were cytosine-2- C^{14} (5.6 mc/mmmole), guanine-2- C^{14} (6.5 mc/mmmole), and adenine-8- C^{14} (10.0 mc/mmmole) obtained from California Corporation for Biochemical Research, and Niche, Inc.

Samples of whole cells or TCA precipitated cells were harvested on membrane filters (Britten *et al.*, 1955). The filters were air-dried at 60–70°C for half an hour and placed in a vial containing 10 ml of a 2, 5-diphenyl oxazole 4 gm/liter (PPO) and 1, 4-bis-2-(5-phenyl oxazolyl) benzene 100 mg/liter (POPOP) solution in toluene. The samples were counted in an automatic Packard Tri-Carb liquid scintillation counter. When it was desired to measure radioactivity remaining in the solution, the cells were removed by centrifugation and samples of the supernatant and resuspended pellet were counted with a thin window gas flow counter. Tests with uracil (McCarthy and Britten, 1962), guanine, and adenine show that, when the total incorporation curve ceases to rise, more than 95 per cent of the added radioactivity has been removed from the medium. In other words nucleic acid bases are not converted to non-utilizable compounds by *E. coli*. Chromatography also shows that greater than 85 per cent of the added radioactivity appears in the nucleotide residues of RNA.

The appearance of radioactivity in the four bases of RNA was determined in each case. For this purpose samples of the cells were treated with 5 per cent TCA, washed twice with water, and hydrolyzed in 0.380 N KOH overnight at 37°C. The KOH was absorbed on fine grain carboxylic ion exchange resin. The nucleotides were then separated by paper strip electrophoresis. The paper was dried and cut into strips which were counted in the liquid scintillation counter.

RESULTS

1. *Cytosine.* The results of an experiment in which 10^{-6} M cytosine-2- C^{14} was supplied to exponentially growing cells are shown in Fig. 1. It is clear that the kinetics of cytosine incorporation are qualitatively similar to the kinetics of uracil incorporation (McCarthy and Britten, 1962). There is an initial rapid incorporation into RNA, and this rate is maintained while exogenous cytosine remains. At the end of this first phase the rate of incorporation into RNA abruptly falls by a large factor. During the second phase (after the exogenous cytosine is exhausted) the radioactivity of the pool falls slowly. A semilog plot (Fig. 2) shows that the radio-

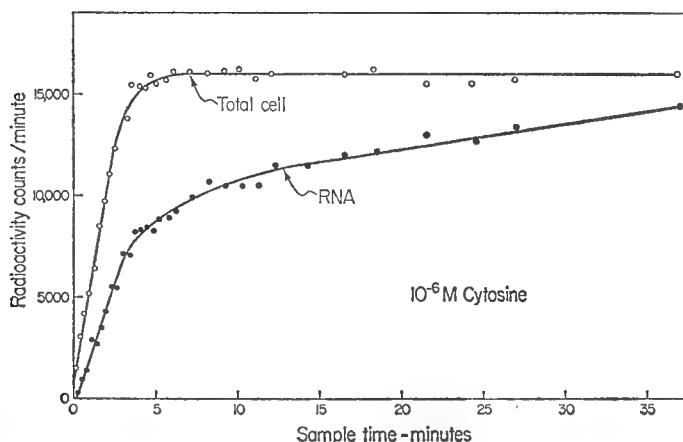


FIGURE 1 Incorporation of 10^{-6} M C^{14} -cytosine by *E. coli* ML 30 growing at 37°C with a generation time of 51 minutes. Cell density 0.5 mg (wet) per ml. Open circles represent radioactivity of total cell samples collected by membrane filtration. Solid circles represent RNA radioactivity; samples collected by membrane filtration after treatment with 5 per cent TCA.

Figs. 1 to 9 are reprinted from *Carnegie Institution of Washington, Yearbook No. 61*, 1961-62.

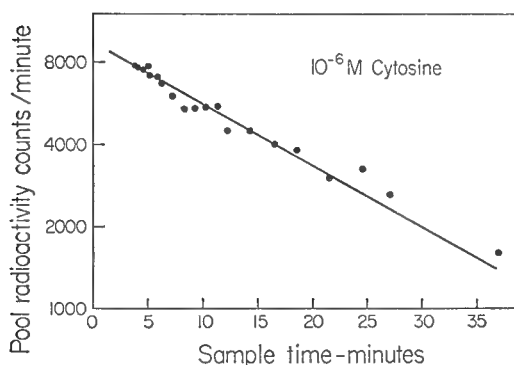


FIGURE 2 Decay of the radioactivity of the C^{14} -cytosine-labeled pool. Data obtained from experiment of Fig. 1 by subtracting the RNA radioactivity from the whole cell radioactivity.

activity of the pool decreases in an approximately exponential fashion. The time constant (decay to $1/e$) is about 21 minutes. This time constant is more than twice that observed for uracil. The results of an experiment at a higher concentration of cytosine (10^{-4} M) are shown on Fig. 3. Here again two phases are observed. Initially C^{14} from cytosine enters the RNA at less than half the final rate. Only after a relatively long period does it achieve its final rate.

The curves shown in Figs. 1, 2, and 3 are just those to be expected on the basis of the schematic diagram, if it is assumed that the time constant is a measure of the quantity of compounds in the pool (S) in relationship to the flow (b). The details of the procedure for the evaluation of the parameters of the model described by the schematic diagram are given in section 4.

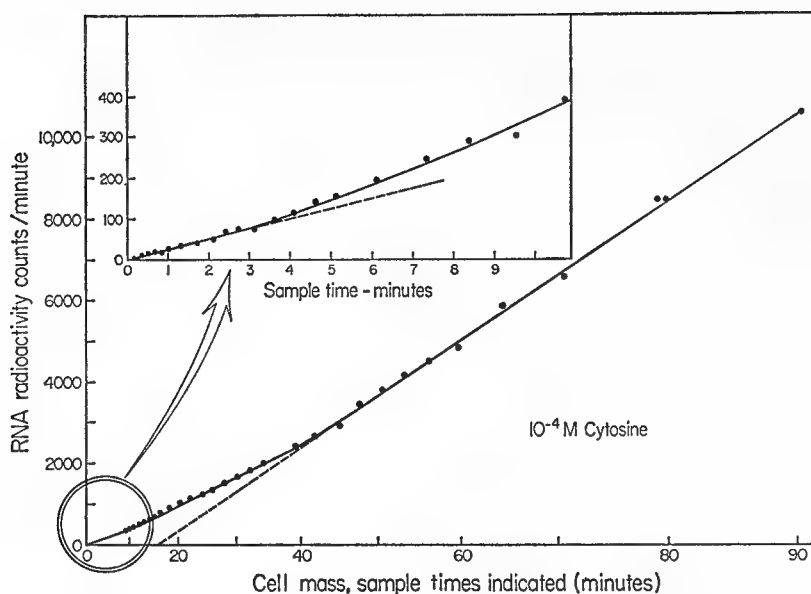


FIGURE 3 Incorporation of 10^{-4} M C^{14} -cytosine by *E. coli*. Initial cell density $\frac{1}{3}$ mg (wet) per ml. RNA radioactivity (TCA-precipitable) plotted against cell mass with sample times indicated. The upper curve represents the data at early times with both scales expanded by a factor of 10. Thus the slopes on the two curves may be directly compared.

The fraction of the total flow bypassing the pool (S) may be estimated from the fraction of the radioactivity (Fig. 1) that has entered the RNA at the time the exogenous cytosine is exhausted. This appears to be about 0.45 as shown in Table II, line 1. The bypass fraction of the flow may also be estimated from the ratio of the initial to ultimate slopes in Fig. 3. The result (Table II, line 2) is consistent with the results of the experiment at low concentration. The shape of the curve on Fig. 3 suggests the presence of components of intermediate time constant in the pool. However, their effect is not apparent on Fig. 2. If such additional components are present the time constant quoted is a weighted average, and the accuracy of the estimate of the bypass flow is reduced. The figure of 0.37 (Table II, row 2) is probably the better figure. Further discussion of the calculation of the bypass flow and time constants is given in section 4.

The time constant estimated here is probably influenced by the appearance of C^{14} from cytosine in uracil compounds in the pool and RNA. Table I shows the results of several measurements of the ratio of the amount of radioactivity in the uridylic acid to that in the cytidylic acid of the RNA. In view of the very considerable inter-conversion between cytosine and uracil compounds it is surprising that the effective time constants of pool (S) measured with the C^{14} -uracil and C^{14} -cytosine are so different. However, comparing lines 4 and 5 with lines 6 and 7 of Table I it appears

TABLE I
INTERCONVERSION OF CYTOSINE AND URACIL COMPOUNDS

Labeled supplement	Competitor	Ratio* of radioactivity of RNA uridylic to RNA cytidylic
C ¹⁴ -cytosine 10 ⁻⁴ M	—	1.4
C ¹⁴ -cytosine 10 ⁻⁴ M	—	1.6‡
C ¹⁴ -cytosine 10 ⁻⁶ M	—	1.7
C ¹⁴ -cytosine 5 × 10 ⁻⁵ M	—	1.6
C ¹⁴ -cytosine 5 × 10 ⁻⁷ M	C ¹² -uracil 5 × 10 ⁻⁵ M	0.6
C ¹⁴ -uracil 5 × 10 ⁻⁵ M	—	1.0
C ¹⁴ -uracil 5 × 10 ⁻⁶ M	C ¹² -cytosine 5 × 10 ⁻⁵ M	1.0

* After the radioactivity was completely incorporated into RNA the cells were washed with 5 per cent TCA, hydrolyzed with alkali, and the nucleotide residues separated by electrophoresis.

‡ In this experiment samples were taken at 10 minute intervals. By 10 minutes the ratio had already reached 1.3 and at 20 minutes had essentially reached its final value.

that C¹²-uracil has an effect on the conversion of C¹⁴-cytosine but C¹²-cytosine does not affect the conversion of C¹⁴-uracil. This lack of symmetry in the competition experiments and the relatively greater conversion of cytosine compounds to uracil compounds indicate the complexity of the interconversion processes.

2. *Guanine*. Fig. 4 shows the results of an experiment in which 10⁻⁶ M guanine was supplied to exponentially growing cells. Here again there is a rapid incorporation into RNA during the first phase when guanine is present externally. During the second phase after the external guanine has been exhausted the radio-

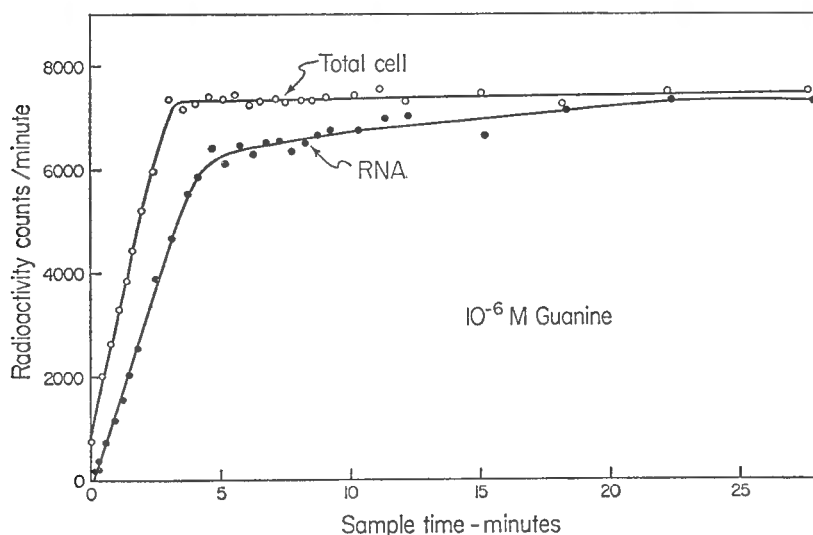


FIGURE 4 Incorporation of 10⁻⁶ M C¹⁴-guanine by *E. coli*. Cell density 0.7 mg (wet) per ml. Open circles represent total cell radioactivity. Solid circles represent RNA radioactivity.

activity of the pool is relatively slowly transferred to RNA. No logarithmic plot of the decay of the guanine pool is presented since the relatively small amount of radioactivity in the pool and the scatter in the points lead to great inaccuracy. Various experiments have given mean time constants between 3 and 6 minutes for the decay of the pool radioactivity after the external guanine is exhausted.

Fig. 5 shows the results of an experiment in which a higher concentration of guanine (10^{-5} M) was supplied. Here a relatively small amount of curvature is observed

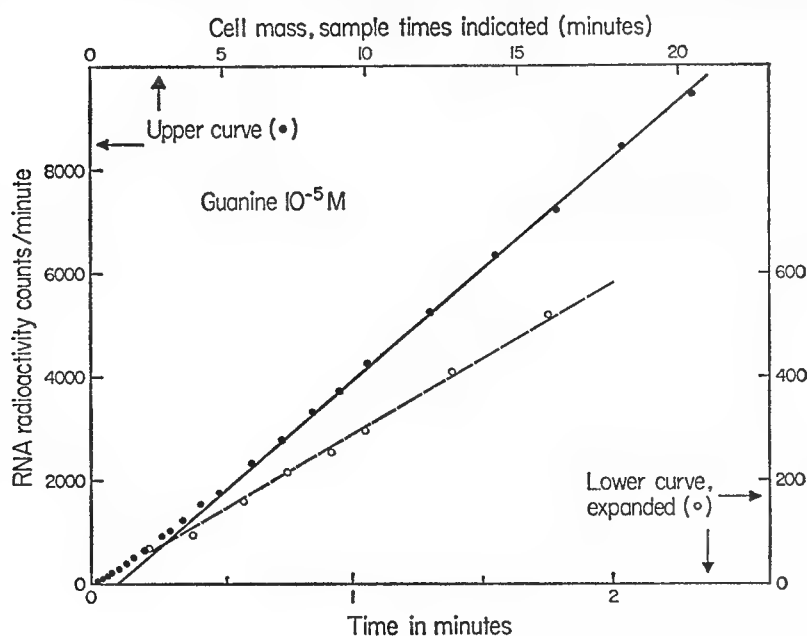


FIGURE 5 Incorporation of 10^{-5} M C^{14} -guanine by *E. coli*. Cell density initially 0.3 mg (wet) per ml. RNA radioactivity plotted against cell mass with sample times indicated. Solid circles refer to upper and left scales. Open circles refer to lower and right scales, both of which are expanded by a factor of 10 so that the initial slope may be directly compared with the final slope.

and a straight line through the points taken after 5 minutes extrapolates to about 1 minute.

The results with guanine are qualitatively similar to those with uracil and cytosine. Two phases in the incorporation curves are observed both at high and at low concentrations. However, the quantitative aspects are quite different. The flow through the pool (S) is relatively small and the time constant is not long (about 3 minutes). The quantity of guanine nucleotides in the pool may be calculated from the time constant and flow. The quantity of guanine compounds may also be estimated directly from the extrapolated time (Fig. 5) to be sufficient to supply the guanine required for 1 minute's growth of the cellular nucleic acid or 7μ moles per gram dry cells. This estimate is valid if there is little exchange between pool guanine compounds

and external guanine and if the conversion to adenine compounds is not too large. C^{14} -guanine does in fact appear only to a slight extent in the adenylic acid of RNA. In three experiments C^{14} -guanine at concentrations of 2×10^{-6} M, 10^{-5} M, and 5×10^{-5} M was allowed to be entirely incorporated into RNA. The ratio of the radioactivity of the adenylic acid of the RNA to that of the guanylic ranged between 0.1 and 0.2.

Fig. 6 shows the results of a "chase" experiment in which C^{14} -guanine ($3.6 \times$

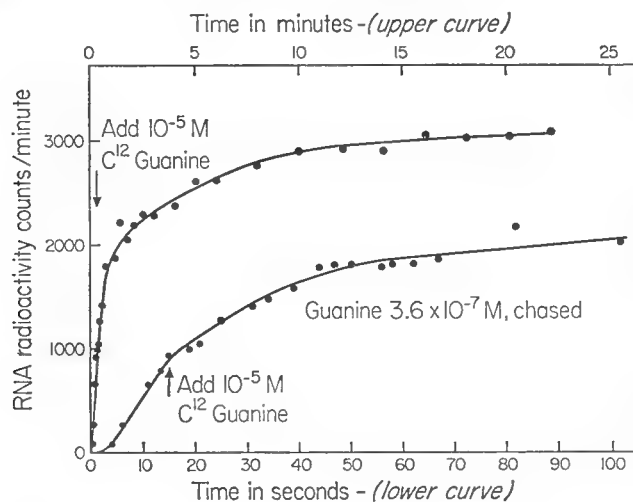


FIGURE 6 Guanine chase experiment. Exponentially growing *E. coli*, cell density 1.8 mg (wet) per ml. Initially C^{14} -guanine (3.6×10^{-7} M) was added. 15 seconds later C^{12} -guanine was added to bring the concentration to 10^{-5} M. Lower curve represents the same data as the upper curve but with time-scale (alone) expanded by a factor of 15.

10^{-7} M) was initially supplied to the cells followed 15 seconds later by 10^{-5} M C^{12} -guanine. This result differs from that obtained with uracil (McCarthy and Britten, 1962) where the specific activity of the uracil passing through the bypass appeared to be diluted almost instantly. In the case of guanine there appears to be a delay of 20 to 30 seconds before the tracer passing through the bypass is completely diluted. There is an instantaneous change in slope to about half that reached during the initial 15 seconds the undiluted tracer was present. This implies the existence in the pool of guanine compounds of a small component with a short time constant. Such a complexity is also suggested by the shape of the RNA curve on Fig. 4 just after the external guanine has been exhausted at 3 minutes, and by the uncertainty in the determination of the decay time constant for the pool of guanine compounds.

3. *Adenine.* Figs. 7 and 8 show the incorporation of C^{14} -adenine at 10^{-7} and 10^{-5} M. Here again the qualitative features are similar to those of the other three bases. Fig. 9 (derived from the experiment of Fig. 7) shows a semilogarithmic

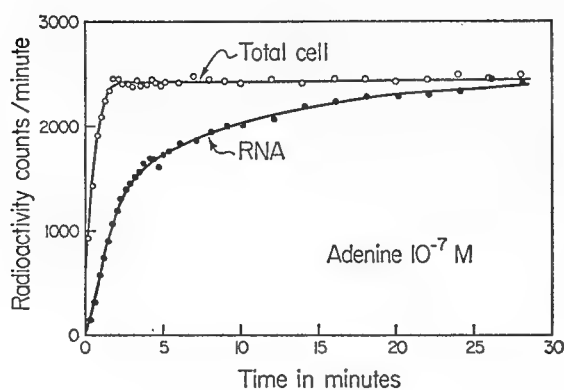


FIGURE 7 Incorporation of 10^{-7} M C^{14} -adenine by growing *E. coli*. Cell concentration $\frac{1}{3}$ mg (wet) per ml. Open circles represent total cell radioactivity. Solid circles represent TCA-precipitable (RNA) radioactivity.

plot of the radioactivity of the pool after the external adenine was exhausted. It is immediately apparent that the decay of the pool radioactivity cannot be represented as a single exponential.

It might be proposed that the shape of the curve on Fig. 8 is influenced by a change in the pool size. The open circles represent the results of an experiment in which 10^{-5} M C^{12} -adenine was added 10 minutes before the tracer. There is no indication of any difference between the two curves on Fig. 8 other than that to be expected from the utilization of a certain fraction of the carrier adenine before the tracer was added. There was, therefore, no measurable expansion of the pool of adenine compounds even at this relatively high concentration of external adenine.

The appearance of radioactivity from C^{14} -adenine in the guanylic acid residues of RNA was measured at two concentrations (10^{-7} M and 10^{-5} M). In both cases the radioactivity of the adenylic residues was about three times that of the guanylic acid residues.

4. *Calculation of the Bypass Flows and Time Constants.* The schematic diagram given in the introduction describes a possible model which is consistent with the observations. The data presented in earlier sections permit the calculation of the parameters of this model. In this section procedures are described for the calculation of the bypass flow ($1-b/c$) and the time constant of the pool (S/b). These parameters have been calculated for each of the four bases from experiments at both high and low concentrations.

An experiment is considered to be at a low concentration if the external supply of labeled base is exhausted before the specific activity of the pool (S) has become comparable to the tracer specific activity. In other words the external supply is exhausted before the rate of incorporation of radioactivity has risen significantly above the initial rate.

If the external supply lasts well beyond the time when the final rate of incorporation into RNA has been achieved, little further change in the results occurs as the concentration is increased. Experiments at intermediate concentrations where neither of these conditions are met are more difficult to interpret.

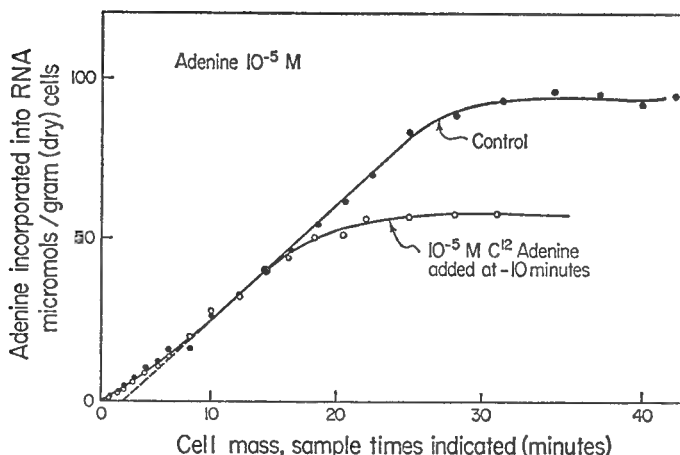


FIGURE 8 Incorporation of 10^{-5} M C^{14} -adenine by growing *E. coli*. Initial cell density 0.42 mg (wet) per ml. Solid circles, control. Open circles 10^{-5} M C^{12} -adenine added 10 minutes before carrier-free C^{14} -adenine.

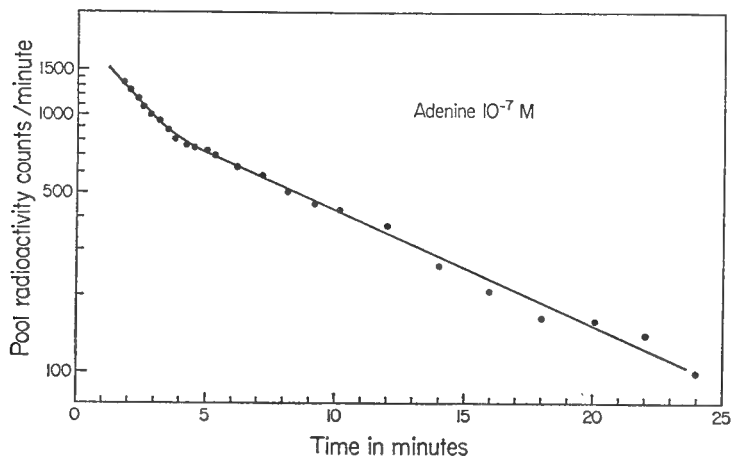


FIGURE 9 Semilogarithmic plot of radioactivity of pool after external adenine was exhausted. Data from Fig. 7.

For experiments at low concentration the bypass flow can be estimated directly from the fraction of the total radioactivity entering the cell that is fixed into the RNA. Thus $(c-b)/c$ listed in the first row in Table II was calculated from the ratio of the slopes of the RNA incorporation curve (TCA precipitable radioactivity) and the total curve. When experiments were available at both 10^{-6} and 10^{-7} M the results agreed.

For experiments at high concentrations the bypass flow was calculated from the ratio of the initial slope of the RNA incorporation curve to its slope after the final rate had been achieved (*i.e.* after S had reached its maximum specific radioactivity). Since there was significant cell growth, all the experiments at high concentration

TABLE II
BYPASS FLOWS AND TIME CONSTANTS

Base supplied	Uracil*	Cytosine	Guanine	Adenine
Fraction of flow in bypass				
Low concentration experiments‡	0.40	0.45	0.74	0.4
High concentration experiments§	0.37	0.37	0.68	0.46
Pool time constant, <i>min.</i>				
Low concentration experiments	10	21	2-6	2-12
High concentration experiments¶	11	24	3.1	4

* Data from McCarthy and Britten, 1962.

‡ From ratio of RNA incorporation rate to total cell incorporation rate.

§ From ratio of initial RNA incorporation rate to final RNA incorporation rate.

|| Time constant of exponential decay of pool radioactivity.

¶ Extrapolated delay time (corrected).

have been plotted against the increase in cell mass. Straight lines on such a plot correspond to constant rates of incorporation per cell. By marking the sample time on the abscissa it becomes possible to estimate directly the time constant by extrapolation (see below).

Row 3 (Table II) lists the time constant of the pool (*S*) estimated on semilogarithmic plots of the pool radioactivity as a function of time from experiments at low concentrations. The time constant estimated in this way is a measure of the ratio of the flow through the pool to the size of the pool if the pool is constant in size. There is no evidence that supplementation with RNA bases expands the nucleotide pools. In those cases that have been tested by "preload" experiments (adenine and uracil) certainly very little if any expansion occurs. The agreement between the data in row 3 and row 4 on Table II supports this conclusion.

Row 4 (Table II) lists the time constant for the increase in the rate of entry of radioactivity into RNA as it rises from its initial rate to its final rate. For this purpose the linear portion of the curve (*e.g.* Fig. 3) at late times is extrapolated until it strikes the time axis giving the effective delay time *T'*. To a sufficiently close approximation the desired time constant is given by $T = R_2 T' / (R_2 - R_1)$ where *R*₁ is the initial slope and *R*₂ the final slope.

DISCUSSION

The evidence presented here and previously (McCarthy and Britten, 1962) shows that the incorporation of the four RNA bases may be represented by the schematic diagram given in the introduction to this paper. The relative bypass flow (*c-b*)/*c*, the size of the pool (*S*), and the time constant of the pool (proportional to *S/b*) vary widely among the four bases. The nucleotide pool appears to contain more than one component and the time course of the decay of the radioactivity of the pool is not always represented by a single exponential. Further, uracil compounds and cyto-

sine compounds are rapidly interconverted. Studies of cytosine (Table I) and uracil (McCarthy and Britten, 1962) show that some of this conversion occurs before entering the pool (S).

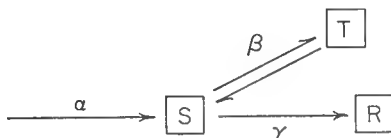
The schematic diagram would obviously grow in complexity if these features were explicitly indicated on it. It is clear that a very large number of significant steps have been ignored or briefly symbolized. As a result the question must be raised as to whether the central feature of the diagram—the existence of a bypass around the large pool—is indeed supported by the evidence.

In the first place it is clear that the evidence rules out models of the following type:



where the flow into RNA is just that required for growth. Such models are not consistent with an undelayed entry into RNA of a given fraction of the tracer that enters the cell (*e.g.*, Fig. 1) nor do they give any explanation of the second phase rise in rate observed in experiments at high concentration (*e.g.*, Fig. 3).

The only alternative that has been proposed (Gros *et al.*, 1961) which will explain the qualitative features may be represented by the following diagram, where S represents the nucleotide pool, R represents the stable RNA, and T represents an unstable (but TCA-precipitable) RNA. The flow γ is just that required for the growth of the stable RNA (R). The flow β is due to the turnover of the unstable RNA (T).



It has been proposed that RNA acting as template for protein synthesis might have the property of very rapid synthesis and breakdown (Jacob and Monod, 1961). If the flow (β) due to the turnover of T is very large, then T and S will effectively have the same specific radioactivity at any time. Thus a certain fraction (determined by the relative size of T and S) of the radioactivity entering the cell will appear without delay in TCA-precipitable RNA.

With the necessary assumption that the flow β is very large the previously presented data permit the calculation of both S and T for each of the four bases. The sum of S and T determines the time constants listed in Table II and the ratio $T/(T + S)$ gives the fraction of the tracer which enters the RNA without delay.

It is only necessary to compare the results for guanine with those for cytosine. When this calculation is carried out in detail it is found that this model requires the quantity of cytidylic acid in T to be 2.5 times the quantity of guanylic acid in T . However, the ratio of the guanylic acid to the cytidylic acid in the rapidly labeled RNA is 0.8 (Midgley and McCarthy, 1962).

Further, since all other RNA labeling will be delayed (according to this scheme) by the time constant determined by S plus T , the observed early labeled RNA must all be in the category represented by T . With uracil as tracer, the time constant of the early labeled RNA fraction is about $2\frac{1}{2}$ minutes (McCarthy *et al.*, 1962) and the time constant listed in Table II (from McCarthy and Britten, 1962) for S is 10 minutes.

Finally the early labeled RNA has been resolved into two distinct fractions (Bolton and McCarthy, 1962). About one-third hybridizes with DNA and appears to turn over by degradation with a time constant of $2\frac{1}{2}$ minutes. The remaining two-thirds does not hybridize with DNA to any large extent and has the same nucleotide composition as ribosomal RNA. There is little doubt that this fraction is precursor to ribosomal RNA and is not degraded before entry into the ribosomes. If this is so, the undelayed entry of tracer into this fraction must result from a bypass of the large nucleotide pools. Thus there is at present no reasonable alternative to the existence of a bypass around the nucleotide pools. It is not surprising that the nucleotide pools are bypassed for RNA synthesis, since the principal function of these pools may be other than in their role as nucleic acid precursors. ATP and other compounds act in the energy transport system, and it is likely that the pool compounds carry out other functions as well.

The relationship of the bypass to the mechanism of pool formation has been previously discussed (McCarthy and Britten, 1962). It was pointed out there that the carrier model (Britten and McClure, 1962) proposed for amino acid pools was consistent with the observations of uracil incorporation. Since the incorporation of the other three bases is in essential respects similar to that of uracil the carrier model gains further support.

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D. Yeast Pools

II.D.1 Kinetics of Formation and Utilization of Metabolic Pools in the Biosynthesis of Protein and Nucleic Acid

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INTRODUCTION

Studies directed toward understanding the flow of material from the medium through various pools of metabolic intermediates into the macromolecules of protein and nucleic acid have been carried out using the yeast-like organism *Torulopsis utilis*. This paper describes first the distribution of tracer carbon and phosphorus among the chemical fractions of the cell during steady-state conditions. This description is followed by a section devoted to a study of the kinetic interrelationship observed among these fractions (a) in the formation of metabolic pools and (b) in the transfer of pool material for macromolecule synthesis. Finally there is a description of the transfer kinetics of individual pool amino acids. The experimental results permit the distinction between endogenous metabolic pools as essential intermediates for macromolecule synthesis and reservoir pools¹ not on the main line of synthetic events. They also indicate that the non-protein amino acids are not free in the cell but rather that they are adsorbed to larger molecules and the adsorption sites are intimately connected with the process of protein synthesis.

RESULTS

Distribution of carbon and phosphorus among the chemical fractions of the cell

After cells have grown exponentially for many generations they reach a steady state in which their cellular composition remains constant. Fig. 1 shows the distribution of phosphorus and carbon among the chemical fractions of the cells during such exponential growth. The rate of formation of each fraction is shown to be directly proportional to the growth rate. In this type of experiment a small inoculum of exponentially growing cells was added to C medium* containing $^{32}\text{PO}_4^{---}$ or ^{14}C -fructose. Twenty-ml samples of the culture suspension were removed at intervals, the cells washed, and chemically fractionated by a modification of the SCHNEIDER method^{2,3}.

The cold TCA-soluble fraction. The steady-state distribution of carbon and phosphorus among the chemical fractions of the cell is shown in Table I. The trichloroacetic acid (TCA)-soluble fraction contains 30% of the phosphorus of the cell. When ^{14}C -fructose is used as the tracer, this fraction was shown to contain 13% of

* C medium: 2 g NH_4Cl , 6 g Na_2HPO_4 , 3 g KH_2PO_4 , 3 g NaCl , 0.010 g Mg as MgCl_2 , 0.026 g S as Na_2SO_4 , 100 ml 10% fructose, and 900 ml distilled H_2O .

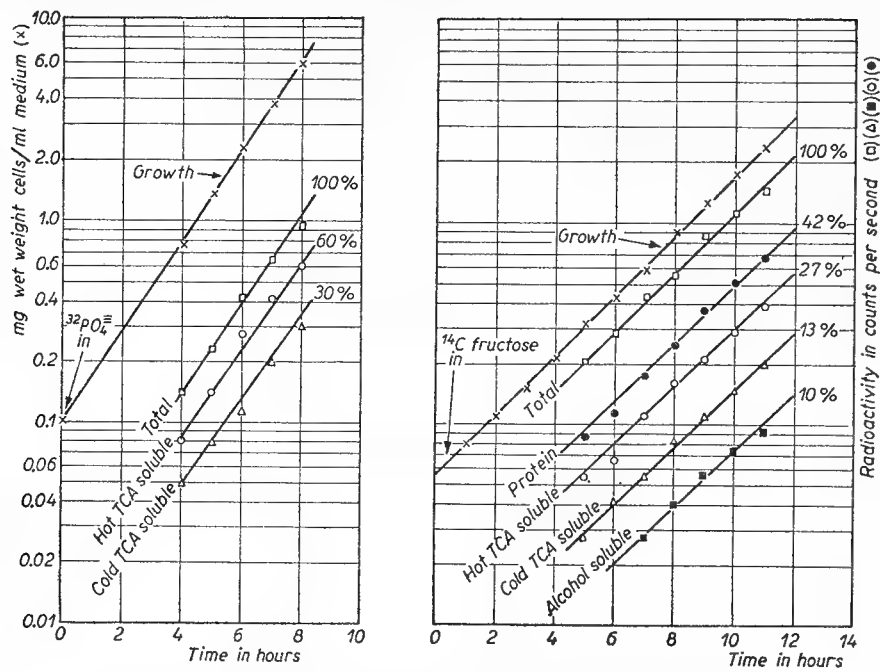


Fig. 1. Steady-state distribution of phosphorus and carbon among the chemical fractions of the cell.

TABLE I

DISTRIBUTION OF CARBON AND PHOSPHORUS AMONG THE CHEMICAL FRACTIONS OF THE CELL *

Fraction	Quantity of carbon (mg per g dry cells)	Quantity of phosphorus (mg per g dry cells)
Total	430	28.0
Cold TCA-soluble	56	8.2
Alcohol-soluble	43	2.7
Hot TCA-soluble	116	16.4
TCA-precipitable	181	—
Percentage accounted for	92	98

* Data obtained from cells growing exponentially in C medium containing ^{14}C -fructose or $^{32}\text{PO}_4$ ---

the total carbon. About 90% of the carbon of this fraction is contained in amino acids with the distribution as shown in Table II.

Chromatographic examination of the unhydrolyzed TCA-soluble fraction obtained from cells grown in $^{32}\text{PO}_4$ --- show that the ^{32}P is distributed among at least 7 separate spots. About 80% of the radioactivity appears in a region with the R_F characteristic of inorganic phosphate, two regions absorb ultraviolet light and have R_F 's corresponding to adenosine triphosphate (ATP) and adenosine monophosphate (AMP). The ratio of radioactivity for these two regions was approximately 3:1 with the ^{32}P content of the ATP region accounting for less than 10% of the phosphorus of the TCA-soluble fraction.

The alcohol-soluble fraction. The alcohol-soluble fraction consists of lipids containing 10% of the total carbon and phosphorus.

TABLE II
DISTRIBUTION OF RADIOCARBON AMONG THE AMINO ACIDS
CONTAINED IN THE TCA-SOLUBLE FRACTION*

Component	Radioactivity in counts per second	Quantity of carbon (mg per g dry cells)
Total radioactivity in fraction	70.4	56
Glutamic acid	22.0	17.4
Alanine	11.0	8.7
Isoleucine-leucine	10.5	8.4
Arginine	5.6	4.5
Valine	4.9	3.9
Lysine	2.1	1.7
Glycine	1.6	1.3
Cyst(e)ine**	1.0	0.8
Aspartic acid	0.5	0.4
Threonine	0.5	0.4
Proline	0.5	0.4
Serine	0.4	0.3
Percentage accounted for	87	

* Data obtained from cells growing exponentially in C medium containing ^{14}C -fructose.

** The expression cyst(e)ine is used to represent either cysteine or cystine.

The hot TCA-soluble fraction. Extraction with hot TCA removes almost all of the remaining phosphorus and 27% of the total carbon. There is 3.4 times as much carbon and about twice as much phosphorus as is required for the cell's nucleic acid, therefore other components must be present in this fraction.

The hot TCA extract obtained from cells growing exponentially in a medium containing ^{14}C -fructose was chromatographed (see ref.², p. 41) and the radioactivity of the isolated purines quantitatively measured. The carbon content of the nucleic acid was calculated from these data to be 29% of the total carbon of the hot TCA-soluble fraction assuming a statistical tetranucleotide composition for the yeast nucleic acids⁴. The remaining carbon was found in two regions on the chromatograms (a) in a streak originating at the start and moving along the base of the chromatogram with the *tert.*-butyl alcohol/HCl solvent and (b) in a region having an R_F characteristic of fructose and glucose. Each region contained about one-third of the carbon of the hot TCA-soluble fraction. Upon elution and further hydrolysis of the material in the streak with 6 *N* HCl for 4 hours and chromatography of the products, it was found that 3% of the ^{14}C was distributed among amino acids and the remainder moved to the front in the *tert.*-butyl alcohol/HCl solvent.

Nucleic acid phosphorus was determined from cells growing exponentially from a light inoculum in a medium containing $^{32}\text{PO}_4^{---}$ with known specific radioactivity. After fractionation, the hot TCA-soluble fraction was hydrolyzed 1 hour with 1 *N* HCl at 90° C. Chromatograms of this fraction were radioautographed and the nucleic acid components were eluted from the chromatogram, their ultraviolet absorption spectra measured quantitatively, and the specific radioactivity determined for both the cytidylic and uridylic acids. The phosphorus content of the nucleic acids was calculated from these data (again assuming a statistical tetra-nucleotide composition for the yeast nucleic acid) and the value obtained (8 mg P per g dry weight of cells) accounted for only half of the phosphorus of the hot TCA-soluble fraction.

TABLE III
DISTRIBUTION OF RADIOCARBON AMONG THE PROTEIN AMINO ACIDS*

Component	Radioactivity in counts per second	Quantity of carbon mg per g dry cells	Quantity of compound μmoles per g dry cells
Isoleucine-leucine	48.8	56.6	785
Lysine	38.6	45.0	625
Glutamic acid	33.2	38.4	640
Aspartic acid	31.6	36.5	762
Phenylalanine	28.2	32.5	301
Valine	26.6	30.7	512
Alanine	19.8	25.0	695
Threonine	18.8	21.9	455
Serine	18.6	21.6	600
Proline	14.7	17.2	287
Tyrosine	13.1	15.3	142
Arginine	13.0	15.1	210
Glycine	10.1	11.7	488
Percentage accounted for	85		

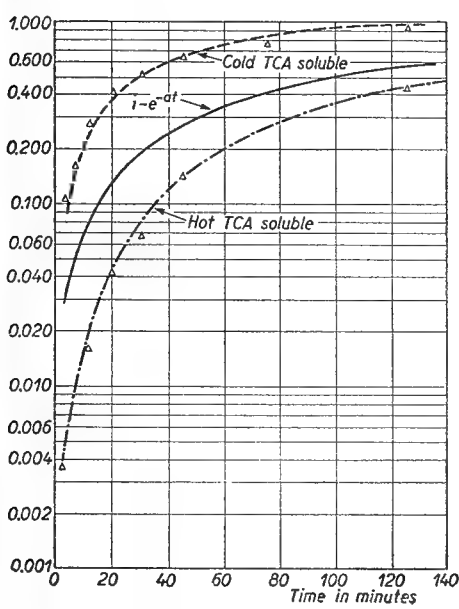
* Data obtained from cells growing exponentially in C medium containing ¹⁴C-fructose.

The identity of the non-nucleic acid carbon and phosphorus in this fraction has not been established.

The TCA-precipitable fraction. The TCA-precipitable fraction contains the balance of the carbon (42%) as cellular proteins. The distribution of ¹⁴C in the amino acids of this fraction is shown in Table III and is markedly different from that obtained in the cold TCA-soluble fraction. Only traces of phosphorus are present in the TCA-precipitable fraction.

Kinetics of incorporation of tracers by exponentially growing cells

Rates of incorporation of ³²P. Fig. 2 shows the rates of incorporation of ³²P into the



cold TCA-soluble and the hot TCA-soluble fractions of the cell as a function of time after the addition of carrier-free ³²PO₄--- to an exponentially growing culture of *T. utilis*. The curves describe the approach to the equilibrium condition in which the phosphorus of the different fractions has the same specific radioactivity (see ref. ², app. 1). The rate constants are *a* and *b*, and *t* is time.

Fig. 2. Rates of incorporation of ³²P into the cold and hot TCA-soluble fractions of the cell after the addition of ³²PO₄--- to an exponentially growing culture of *T. utilis*. The ordinate is in arbitrary units of radioactivity per gram of cells. The experimental values are fitted to the theoretical curves at one point. Carrier ³¹PO₄--- was present in the medium during the course of the experiment.

$$\text{---} (1 - e^{-bt}) = (1 - e^{-0.0231 t})$$

$$\text{---} (1 - e^{-at}) = (1 - e^{-0.0077 t})$$

$$\text{---} 1/(b-a) \{b(1 - e^{-at}) - a(1 - e^{-bt})\}$$

$$\Delta \Delta \Delta \text{ Experimental points.}$$

The curve describing the incorporation of ^{32}P into the TCA-soluble fraction appears to be a simple exponential function with a coefficient three times the growth rate. This rapid incorporation in excess of the growth rate would be required if the TCA-soluble pool (30% of the cell's phosphorus) incorporates phosphorus in order to form the pool and provide for the subsequent transfer of phosphorus to the hot TCA-soluble fraction (60% of the total phosphorus).

This scheme is shown in Fig. 3.

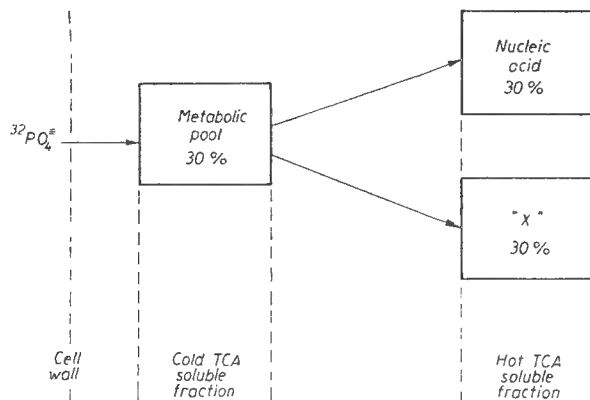


Fig. 3. Explanation in text.

The theoretical curve of incorporation of ^{32}P into the hot TCA-soluble fraction according to the above scheme is also shown in Fig. 2. This curve labeled

$$1/(b-a) [b(1-e^{-at}) - a(1-e^{-bt})]$$

describes the theoretical rate of incorporation of ^{32}P into the hot TCA-soluble fraction assuming that all of the ^{32}P was derived from the TCA-soluble pool. The agreement of the experimental data with the theoretical curve is excellent. It has also been assumed that there is no loss of incorporated phosphorus to the medium during the course of the experiment.

Transfer of pool phosphorus to end products. Since the incorporation of phosphorus into the cold TCA-soluble pool precedes the synthesis of the other fractions, it is possible to prepare cells containing most of the ^{32}P in the metabolic pool. This is done by briefly immersing exponentially growing cells in medium containing ^{32}P and transferring the cells, after washing, to medium containing non-radioactive phosphorus. The flow of the labeled pool phosphorus to the other fractions of the cell can then be followed. As shown in Fig. 4 the ^{32}P rapidly leaves the TCA-soluble fraction and for the most part appears in the hot TCA-soluble fraction. The phospholipids, containing about 10% of the phosphorus of the cell, also receive some of the pool ^{32}P . There appears to be little or no loss of the labeled ^{32}P from the cell to the medium. Interrelations between the nucleic acid phosphorus and the unidentified phosphorus ("X") of the hot TCA-soluble fraction have not been established.

Rates of incorporation of ^{14}C from ^{14}C -fructose. Similar kinetics of formation and transfer are observed when ^{14}C -fructose is used as the tracer. Fig. 5 shows the incorporation of ^{14}C into the metabolic pool (TCA-soluble fraction) at a rate 5.2 times the growth rate.

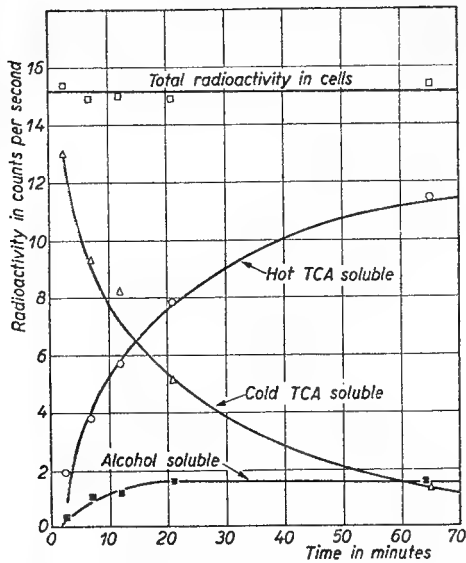


Fig. 4. The transfer of labeled phosphorus from the cold TCA-soluble fraction to the hot TCA-soluble and alcohol-soluble fractions of the cell. Exponentially growing cells were briefly immersed in medium containing $^{32}\text{PO}_4$ and transferred after washing to non-radioactive medium.

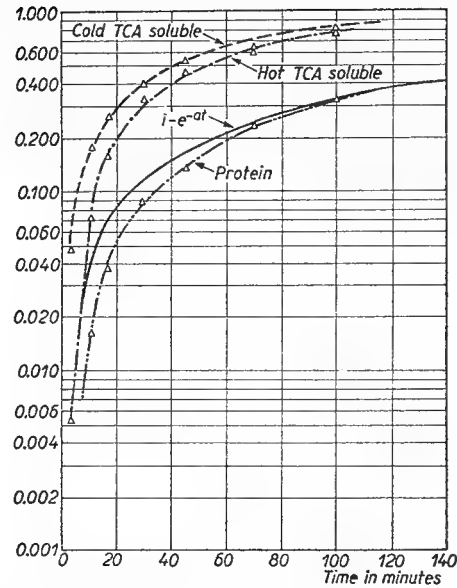


Fig. 5. Rates of incorporation of ^{14}C into chemical fractions of the cell after the addition of ^{14}C -fructose to an exponentially growing culture of *T. utilis*. The rate of incorporation into the cold and hot TCA-soluble fraction is faster than $i-e^{-at}$ whereas ^{14}C incorporation into protein is slower. Carrier ^{12}C -fructose was present in the medium during the course of the experiment. Experimental values are fitted to the theoretical curves at one point. ——— $(1-e^{-bt}) = (1-e^{-0.0173t})$; ——— $(1-e^{-at}) = (1-e^{-0.0033t})$; ····· Hot TCA-soluble fraction; ····· TCA-precipitable; $\Delta \Delta \Delta$ Experimental points.

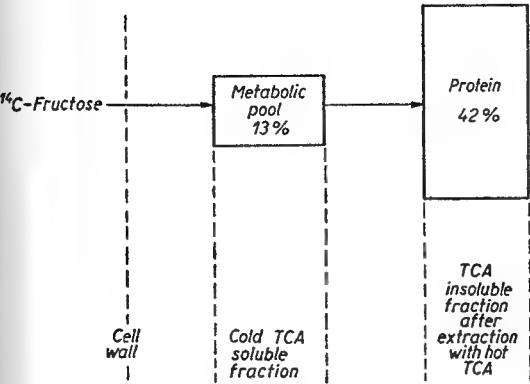
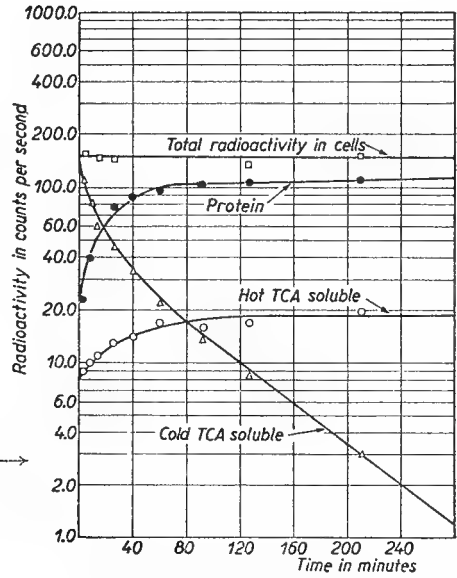


Fig. 6. Explanation in text.

Fig. 7. The transfer of ^{14}C from the cold TCA-soluble fraction to the hot TCA-soluble and cold TCA-precipitable fractions of the cell. Exponentially growing cells were briefly immersed in medium containing ^{14}C -fructose and transferred after washing to non-radioactive medium.



The incorporation of ^{14}C into the proteins of the cell is slower than the growth rate and must therefore be derived from some preformed unlabeled pool during the earlier periods of the experiment. If the simplified model shown schematically in Fig. 6 were correct then the expected rate of formation of the intermediate pool would be 4.25 times the growth rate.

It is not surprising that the observed rate of formation exceeds the expected rate of formation based on the simplified model shown above, since it is well known that purines and pyrimidines derive some of the carbon from amino acids. Indeed, we have observed the transfer of some of the pool carbon both to the hot TCA-soluble fraction and to the alcohol-soluble fraction of the cell.

Transfer of cold TCA-soluble pool carbon to end products. Additional information can be obtained by following the transfer of the intermediate pool carbon to the end products of the cell. When the TCA-soluble pool carbon is prelabeled by briefly immersing exponentially growing cells in medium containing ^{14}C -fructose and then transferring immediately to medium containing ^{12}C -fructose, most of the ^{14}C is contained in the intermediate pool. Fig. 7 shows the results of such an experiment in which more than 70% of the incorporated ^{14}C was initially bound in the TCA-soluble pool.

There is a rapid loss of pool radiocarbon, 50% being lost in the first 20 minutes. The major portion of the carbon appears in the proteins and to a lesser extent in the hot TCA-soluble fraction and the alcohol-soluble fractions. Very little if any of the labeled carbon is lost to the medium. Fig. 7 shows that the rate of flow of carbon from the cold TCA-soluble fraction is not constant. There appear to be at least two rates of transfer of carbon from this pool indicating that the pool is complex.

Kinetics of hot TCA-soluble fraction. It has been observed that the incorporation of ^{14}C -fructose carbon into the hot TCA-soluble fraction is erratic, particularly at the beginning or the end of the exponential growth phase. As shown in Fig. 5 the rate of ^{14}C incorporation may be very rapid, exceeding the growth rate. Furthermore, Fig. 7 shows that only a small part of the cold TCA-soluble pool radiocarbon is transferred to the hot TCA-soluble fraction, the major recipient being the proteins of the cell. Finally, the rate of formation of the cold TCA-soluble pool is 5.2 times the growth rate, with most of the carbon of this pool being required for the proteins. If in addition all of the carbon of the hot TCA-soluble fraction as well as that of the proteins came directly from this pool, the rate of formation of the cold TCA-soluble pool would have to be at least 6.3 times the growth rate. Thus the hot TCA-soluble fraction appears to be derived in part from the cold TCA-soluble fraction and in part from the exogenous fructose.

The hot TCA-soluble fraction is a complex one containing carbon and phosphorus other than nucleic acid. Experiments were therefore carried out to investigate the kinetics of formation and utilization of an individual nucleic acid component.

For this purpose ^{14}C -adenine was added to an exponentially growing culture of *T. utilis*. The incorporation into the cold TCA-soluble fraction was rapid as shown in Fig. 8. The rate of incorporation of ^{14}C into this fraction was about 8 times the growth rate and most of the incorporated carbon was initially in the cold TCA-soluble pool. Equilibrium was rapidly reached and was then maintained throughout the experiment. The rate of appearance of radiocarbon in the hot TCA-soluble fraction was slower than the growth rate initially but rapidly approached this rate as the cold TCA-soluble pool reached equilibrium.

The incorporated radiocarbon in the hot TCA-soluble fraction was identified as adenine and guanine by chromatography. These results indicate that during exponential growth adenine carbon is rapidly incorporated into the cold TCA-soluble pool and in turn is transferred to the adenine and guanine of the nucleic acid of the cell. These bases in the nucleic acid are stable end products in the utilization of adenine

carbon. The rate of incorporation into nucleic acid cannot exceed the growth rate in exponentially growing cells.

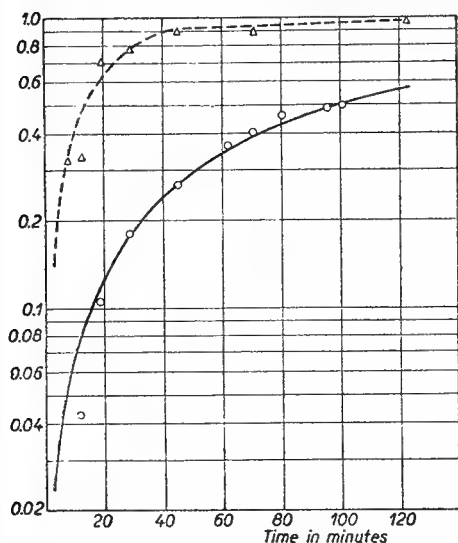


Fig. 8. Rate of incorporation of ^{14}C into the cold TCA-soluble and hot TCA-soluble fractions of the cell after the addition of ^{14}C -adenine to an exponentially growing culture of *T. utilis*. The experimental values are fitted to the theoretical curves at one point. Carrier ^{12}C -adenine was present in the medium during the course of the experiment. — ($1 - e^{-at}$); Δ cold TCA-soluble fraction; \circ hot TCA-soluble fraction.

acid bases. Chromatographic examinations were therefore made of the hot TCA-soluble fractions obtained from exponentially growing cells during the early period following the addition of ^{14}C -fructose to the medium. The radiocarbon first appeared in the fraction shown in Fig. 9 as "Y", and in the non-nucleic acid sugar. The nucleic acid bases were observed to become radioactive at a much slower rate. These data show that one of the rapidly formed constituents in the hot TCA-soluble fraction is "Y" and that "Y" is formed from exogenous ^{14}C -fructose.

The non-nucleic acid carbon of the hot TCA-soluble fraction appears to be contained in a reservoir pool and not as a direct intermediate for end-product formation. When the contents of this pool are low (for reasons unknown) rapid replenishment at rates in excess of the growth rate can be observed (Fig. 5). On the other hand when this reservoir pool is filled the incorporation of ^{14}C -fructose is characteristic of incorporation into an end product. This situation is parallel to that observed in the kinetics of formation and transfer of the glutathione sulfur which can serve as a sulfur reservoir when exogenous sulfur is not available (see ref. ², chap. 18). The role the non-nucleic acid carbon plays in cellular metabolism or macromolecule synthesis is not known.

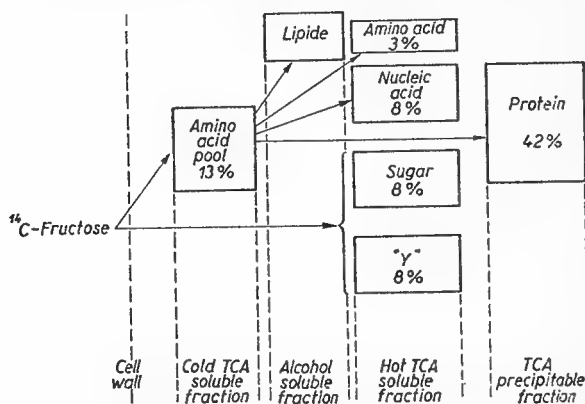


Fig. 9. Explanation in text.

As adenine carbon is incorporated into the adenine and guanine of the hot TCA-soluble fraction at a rate approximately that required for growth, the rapidly incorporated ^{14}C from ^{14}C -fructose must be in other substances than these nucleic

The kinetics of transfer of the carbon of individual amino acids from pool to protein

It is surprising that the rate of formation of the cold TCA-soluble pool can be approximated by a simple exponential function (Fig. 5). This pool contains many amino acids in widely differing amounts (Table II). These amino acids furnish carbon for the constitutive proteins of the cell, but the amino acid distribution found in the proteins (Table III) varies markedly from that found in the pool. Clearly certain of the protein amino acids have large quantities of their counterpart in the TCA-soluble pool while for others only traces are detectable. The turnover rate of specific amino acid carbon from pool to protein must then vary depending upon the quantity of that amino acid in the pool and the quantity of that amino acid required for protein formation.

Accordingly, the transfer of the carbon from the individual amino acids of the pool to protein has been investigated. ^{14}C -fructose was added to a culture of exponentially growing cells for 10 minutes in order to label the precursor pool preferentially. These cells were then washed in C medium and transferred to fresh medium containing ^{12}C -fructose as the sole carbon source. Samples were removed at various intervals, the cells fractionated and two-dimensional chromatograms made of aliquots of hydrolysates of the cold TCA-soluble and precipitable fractions. The rates of loss of radiocarbon from several amino acids in the TCA-soluble fraction are shown in Figure 10.

Loss of radiocarbon from pool amino acids. The exponential rates of loss of ^{14}C -alanine and glutamic acid indicate that this loss is a simple process and that there is complete mixing of the radioactive amino acids with the non-radioactive amino acids of the pool. The ^{14}C -arginine of the pool was not initially lost at an exponential rate but as shown in Fig. 10, increased in radioactivity and then decreased at an exponential rate equal to that observed for the ^{14}C -glutamic acid. These data are characteristic of a more complex process in which pool arginine is an intermediate between pool glutamic acid and protein arginine. It has been previously demonstrated in *T. utilis* that some of the carbon in protein arginine and proline is derived from glutamic acid (see ref. ², chap. 13).

The distribution of ^{14}C among the amino acids in the cold TCA-soluble fraction from cells briefly immersed in ^{14}C -fructose is significantly different from that observed in cells grown for many generations in the labeled medium. Table IV shows the comparative distributions of ^{14}C under these two conditions. During the brief immersion

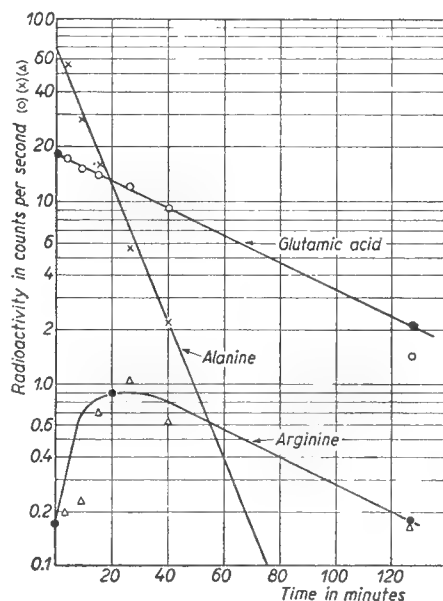


Fig. 10. Time course of ^{14}C concentration in amino acids of the cold TCA-soluble fraction. Data obtained from cells briefly immersed in medium containing ^{14}C -fructose and transferred after washing to non-radioactive medium. (X), (Δ), and (O) are experimental data. Parameters of theoretical curves (unbroken line) were chosen to pass through theoretical points (\bullet).

TABLE IV

DISTRIBUTION OF RADIOCARBON AMONG AMINO ACIDS OF THE COLD TCA-SOLUBLE FRACTION OBTAINED FROM CELLS GROWN IN C MEDIUM CONTAINING ¹⁴C-FRUCTOSE

Amino acid	Period of exponential growth in ¹⁴ C medium	
	10 minutes	4 hours
(radioactivity in counts per second)		
Alanine	34.4	11.0
Glutamic acid	19.1	22.0
Valine	8.8	5.0
Aspartic acid	3.2	0.5
Isoleucine-leucine	2.3	10.5
Arginine	0.1	5.6

period more ¹⁴C was incorporated in alanine than in any other amino acid. The total quantity of alanine in the TCA soluble fraction is less than the total glutamic acid in this fraction (Table II). Fig. 10 indicates that the turnover rate for alanine is about 5 times faster than that observed for the glutamic acid. These turnover rates are dependent upon at least two factors (a) the size of the individual amino acid pools and (b) the specific requirements upon pool constituents for the formation of end products. In some cases these end products are other pool amino acids as well as the material required for protein formation. When end-product requirements are high and the size of the pool small, turnover rates are rapid.

Those pool amino acids which receive their carbon directly from the medium are the first to be labeled. On the other hand it is apparent from Table IV and Fig. 10 that not all of the pool amino acids receive all of their carbon directly from the medium.

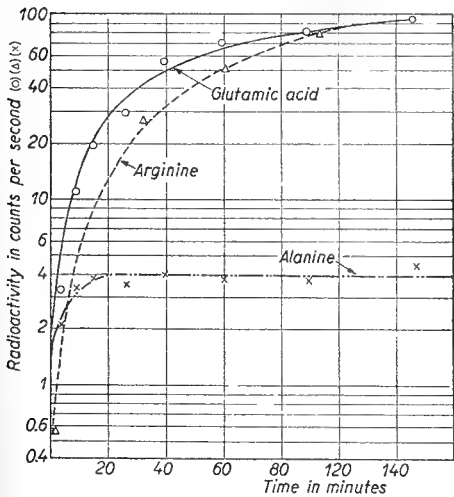


Fig. 11. Time course of ¹⁴C concentration in protein amino acids. Data obtained from cells briefly immersed in medium containing ¹⁴C-fructose and transferred after washing to non-radioactive medium.

Those which are the “heads” of each “family of amino acids” (see ref. ², chap. 20) furnish carbon to the members of their respective families. Such family members are pool intermediates between the family “heads” and protein end products.

Incorporation of pool amino acid carbon into protein. The loss of ¹⁴C from individual amino acids in the metabolic pool described above permits certain predictions regarding the appearance of pool radiocarbon into the amino acids of the proteins. Fig. 11 shows that some of the ¹⁴C derived from pool alanine is rapidly incorporated into the alanine of the proteins. At 30 minutes less than 10% of the initially ¹⁴C-labeled pool alanine remains in the pool (Fig. 10). At this time very little ¹⁴C is therefore available from pool alanine for protein alanine requirements.

The rate of loss of pool ¹⁴C-glutamic acid is about one-fifth that observed for pool alanine and unlike alanine there was still some radioglutamic acid present in the pool at the end of the experiment. The

appearance of ^{14}C -glutamic acid in the protein of the cell (Fig. 11) reflects continued availability of the pool ^{14}C -glutamic acid.

The rate of appearance of ^{14}C into protein arginine is very similar to that observed for glutamic acid. Initially however, since the pool arginine radioactivity is low there is a delay in the appearance of ^{14}C into the arginine of the proteins. In a similar fashion the kinetics of transfer of other pool amino acids were investigated and it was found that for some there was an exponential loss of amino acid carbon and others followed the more complex kinetics observed for pool arginine. No turnover rate of an individual pool amino acid has been found to be slower than twice the growth rate (glutamic acid) and the fastest measured (alanine) corresponds to a rate of formation 10 times the growth rate.

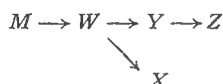
The experimental results described for the formation and utilization of the individual pool amino acids can be quantitatively examined as shown below.

The equations for incorporation of a labeled substance into exponentially growing cells have been previously described (see ref. ², app. 1). These equations, while sufficient for the analysis of data shown in Figs. 2, 5, 8, etc. do not apply when describing the loss of radioactivity from cellular components. Additional equations are therefore necessary.

If a quantity of cells, Q is growing exponentially then

$$Q = Q_0 e^{at} \quad (1)$$

where Q_0 is the original quantity of cells and Q is the number at time t , a is the rate constant. Let W , X , Y , and Z be different compounds derived from a common medium M as shown below



During steady-state growth the relative proportions of W , X , Y , and Z do not change and

$$\begin{array}{l} W = W_0 e^{at} \\ X = X_0 e^{at} \\ Y = Y_0 e^{at} \\ Z = Z_0 e^{at} \end{array} \quad (2)$$

However, these equations are the solutions respectively of the following differential equations

$$\begin{array}{l} dW/dt = k_0 Q - k_1 Q \\ dX/dt = k_2 Q \\ dY/dt = k_3 Q - k_4 Q \\ dZ/dt = k_5 Q \end{array} \quad (3)$$

if it is assumed that the rates of formation and utilization are proportional to the quantity of cells. Differentiating (2) and substituting in (3) gives the relations between the steady-state concentrations W_0/Q_0 , X_0/Q_0 , etc. and the transfer coefficients k_1 , k_2 , k_3 , k_4 , and k_5 .

$$\begin{array}{l} W_0/Q_0 = (k_0 - k_1)/a \\ X_0/Q_0 = k_2/a \\ Y_0/Q_0 = (k_3 - k_4)/a \\ Z_0/Q_0 = k_5/a \end{array} \quad (4)$$

If the cells are grown for a period in a radioactive medium (M^*) and subsequently transferred to a non-radioactive medium at $t = 0$, then the differential equations

which describe the transfer of radioactivity from one compound to another will be

$$\begin{aligned} dW^*/dt &= -k_1 Q W^*/W \\ dX^*/dt &= k_2 Q W^*/W \\ dY^*/dt &= k_3 Q W^*/W - k_4 Q Y^*/Y \\ dZ^*/dt &= k_5 Q Y^*/Y \end{aligned} \quad (5)$$

These equations assume that the quantity of radioactive material transferred is proportional to the specific radioactivity; *i.e.* that there is complete mixing between radioactive and non-radioactive materials of the pool even though the radioactive and non-radioactive material may have entered the pools at different times. By differentiating the equations (6)

$$W^* = W_0 e^{-bt} \quad (a)$$

$$X^* = X_0^* + k_2/k_0 W_0^* (1 - e^{-bt}) \quad (b)$$

$$Y^* = Y_0^* e^{-ct} + \frac{k_3 Q_0}{(c-b)W_0} (e^{-bt} - e^{-ct}) \quad (c) \quad (6)$$

$$Z^* = Z_0^* - \frac{k_5 Q_0 Y_0^*}{Y_0 c} e^{-ct} + \frac{k_3 Q_0}{(c-b)W_0} \left(\frac{1}{b} e^{-bt} - \frac{1}{c} e^{-ct} \right) \quad (d)$$

it can be shown that they are solutions of (5) provided that

$$\begin{aligned} b &= a \left(\frac{k_1}{k_1 - k_0} \right) = k_1 \frac{Q_0}{W_0} \\ c &= \frac{ak_4}{k_3 - k_4} = \frac{k_4 Q_0}{Y_0} \end{aligned} \quad (7)$$

Equation (6a) is appropriate for describing the disappearance of TCA-soluble ^{14}C -alanine or ^{14}C -glutamic acid. Equation (6b) predicts the corresponding appearance of ^{14}C -alanine or ^{14}C -glutamic acid in the protein fraction. Equation (6c) describes the build up and decay of ^{14}C -arginine in the TCA-soluble fraction and (6d) gives the appearance of ^{14}C -arginine in the protein fraction. These equations predict the form of the transfer curves correctly (Fig. 10 and 11) and when the parameters are chosen appropriately the curves are in good agreement with the experimental data.

The success of these equations shows clearly that material is withdrawn from the pools in a random fashion and rules out any "assembly line" or "extrusion" model wherein the pool material would be withdrawn on a "first in, first out" basis.

Isotopic competition studies. When *T. utilis* is grown in C medium containing both ^{14}C -fructose and ^{12}C -amino acids, exogenous amino acids reduce the incorporation of radiocarbon into the proteins of the cell (see ref. ², chap. 13). Since the flow of carbon from fructose in the medium to the proteins of the cell was experimentally demonstrated to pass through the amino acid pool, the effects of exogenous amino acids upon pool formation and utilization were kinetically investigated.

An exponentially growing culture of *T. utilis*, immersed for 10 minutes in medium containing ^{14}C -fructose, was washed, divided into two equal fractions, and quickly transferred to fresh C medium containing ^{12}C -fructose. More than 70% of the total ^{14}C in the cells was found in the TCA-soluble fraction. To one culture was added a mixture of ^{12}C -amino acids at concentrations as shown in Table V. Samples were removed from both cultures at intervals following this transfer.

Table V shows the rapid loss of ^{14}C from the TCA-soluble fraction for both the control culture and the culture containing the exogenous ^{12}C -amino acids. No difference could be detected in the loss rates in the two cultures. Both cultures continued

TABLE V

EFFECT OF EXOGENOUS ^{12}C -AMINO ACIDS ON THE LOSS OF RADIOCARBON FROM THE TCA-SOLUBLE FRACTION IN EXPONENTIALLY GROWING *T. utilis*

Time in minutes	mg wet weight per ml medium	Total radioactivity in TCA-soluble fraction	
		^{13}C -amino acids present*	No amino acids present
(radioactivity in counts per second)			
5	0.59	20.8	20.8
17	0.67	12.2	12.4
35	0.82	8.2	8.3
62	1.00	4.7	4.9
92	1.35	2.9	3.0

* ^{12}C -amino acids present as competitors (mg per ml medium): aspartic acid 0.75; glutamic acid 0.75; alanine 0.50; valine 0.25; glycine 0.25; lysine 0.25; serine 0.25; arginine 0.19; leucine 0.19; proline 0.12; threonine 0.12; isoleucine 0.12; methionine 0.10; tyrosine 0.10.

TABLE VI

EFFECT OF EXOGENOUS ^{12}C -AMINO ACIDS ON THE APPEARANCE OF RADIOCARBON IN PROTEIN AMINO ACIDS

Protein amino acid	Radioactivity in protein amino acids	
	^{12}C -amino acids present*	No amino acids added
(radioactivity in counts per second)		
Glutamic	7.1	7.0
Threonine	6.5	6.8
Aspartic	13.0	11.5
Arginine	4.5	4.2
Alanine	7.0	6.5
Glycine	8.2	8.3

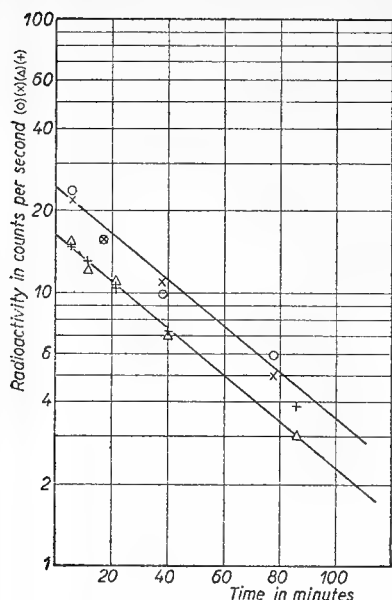
* ^{12}C -amino acids present at concentrations as shown in Table V.

to grow exponentially at the same rate as observed prior to and during the labeling period.

Measurements were made of the radioactivity contained in individual amino acids of the proteins obtained from cells at the end of the experiment. Table VI shows that no significant differences exist in the distribution of ^{14}C in these amino acids of the two cultures.

This experiment demonstrates that the exogenous ^{12}C -amino acids have no effect upon the transfer of pool radiocarbon to the protein amino acids. The competitive effects of exogenous amino acids observed when both ^{14}C -fructose and ^{12}C -amino acids are present during the labeling period therefore occur either before, or at the time the amino acids enter the metabolic pool. Those amino acids already associated in the pool are not affected by the presence of the exogenous competitors.

More direct evidence confirming this conclusion may be obtained by following the fate of the carbon of a single pool amino acid. When exponentially growing cells briefly immersed in medium containing ^{14}C -glutamic acid were washed and transferred to fresh C medium containing no radioactive supplement, there was a rapid loss of the radioglutamic acid of the pool as shown in Fig. 12. This radiocarbon eventually appeared as protein ^{14}C -glutamic, -arginine, and -proline. Fig. 12 shows that the



same rate of loss of pool ^{14}C -glutamic acid occurs whether ^{12}C -arginine is present or not. There is no detectable alteration in the flow of pool ^{14}C -glutamic acid when the exogenous competitor is present. Similar evidence was obtained using ^{14}C -aspartic and ^{12}C -threonine, also shown in Fig. 12.

Fig. 12. Loss of ^{14}C -glutamic and ^{14}C -aspartic acid carbon from the cold TCA-soluble fraction. Data obtained from cells (approximately 1.0 mg wet weight per ml medium at $t = 0$) briefly immersed in medium containing either ^{14}C -glutamic or aspartic acid and transferred after washing to non-radioactive medium containing amino acid supplements (1.0 mg per ml medium) or to non-radioactive medium without amino acid supplements. ○ Pool ^{14}C aspartic; × Pool ^{14}C aspartic + exogenous ^{12}C threonine; △ Pool ^{14}C glutamic; + Pool ^{14}C glutamic + exogenous ^{12}C arginine.

DISCUSSION AND CONCLUSIONS

GALE⁵ and TAYLOR⁶ have shown that certain gram-positive organisms contained considerable quantities of easily extractable amino acids. The work of HALVORSON AND SPIEGLEMAN demonstrated the presence of "free amino acid pools" in *Saccharomyces cerevisiae* and showed that such pools could be used for the synthesis of induced enzymes^{7,8}. The investigations reported here have shown that in *T. utilis* the amino acid pool is not simply a mixture of "free amino acids". Amino acids are concentrated in the pool in excess of the free amino acid content in the medium. Once incorporated, exogenous amino acids have little or no effect upon the internal conversions of these pool amino acids or upon the rate of their incorporation into proteins. Such behavior could be accounted for by the formation of an amino acid-R group complex². The present kinetic studies reveal some of the properties of the amino acid-R group complex and indicate the role such an organized pool of amino acids plays in the synthesis of nucleic acid and protein.

In *T. utilis* the metabolic pool of amino acids appears to be a direct intermediate in the synthesis of proteins from exogenous fructose. The carbon of endogenously formed amino acids was shown to be directly incorporated into the metabolic pool prior to its appearance in the proteins of the cell. The same was true for exogenous amino acids and in both cases the amino acids transferred to the proteins have the same specific radioactivity as the amino acids of the pool.

The results obtained on the transfer kinetics of pool amino acid carbon in *T. utilis* are significantly different from those observed in the utilization of reservoir pools of sulfur in *E. coli*^{1,2}. In *E. coli* glutathione was shown to act as a sulfur reservoir which was drawn upon only during period of sulfur privation. When adequate external sulfur was available the exogenous sulfur was incorporated into protein without passing through the glutathione pool. Removal of exogenous sources caused the cells to utilize internal glutathione sulfur as long as it was available. Such kinetic studies allow

the distinction between endogenous metabolic pools essential as intermediates for macromolecule synthesis and reservoir pools not on the main line of synthetic events.

Furthermore, metabolic transformations have been observed among the individual amino acids of the pool. During incorporation of ^{14}C from the fructose of the medium, the "heads" of each "family of amino acids" become radioactive before the other family members; later when the source of ^{14}C is removed from the medium these members gain in radioactivity at the expense of the family "head". These transformations are not affected by the presence of external amino acids. It can be concluded therefore that the amino acid-macromolecule complex once formed is not broken during the series of reactions required for the conversion of one pool amino acid to another.

Finally exogenous amino acids do not interfere with the conversion of pool amino acids to protein amino acids even though they can prevent formation of pool amino acids from fructose. Again it appears that the amino acid-R group complex is not broken prior to the incorporation of pool amino acid into protein. These results suggest that there is an intimate connection of the amino acid complex with protein formation.

Fig. 13 represents a type of model which is necessary to explain the observed competition between exogenous amino acids and fructose, as well as the lack of competition between exogenous amino acids and pool amino acids.

When the cell is growing with fructose as the sole carbon source, certain amino acids such as aspartic and glutamic acids, alanine, and serine (the "family heads") are rapidly formed and adsorbed on sites on the macromolecule-R. Not all of the pool amino acids will be adsorbed on a single R-group nor will all the R-groups be identical. Glutamic acid can be adsorbed on several types of sites indicated as "A", "P", and "G" in Fig. 13. When adsorbed on site "G" it is subsequently incorporated into the protein as glutamic acid. When adsorbed on site "A" it must be transformed to arginine before it is incorporated into the protein and the conversion of glutamic acid on "P" to proline is necessary before it is incorporated.

If exogenous amino acids are also present in the medium they will compete with the endogenously formed amino acids (from fructose) for empty amino acids sites on R, but they will not displace amino acids already adsorbed. For example, exogenous ^{12}C -arginine could fill any unoccupied "A" site and thus exclude ^{14}C derived from ^{14}C -fructose from reaching protein arginine. However, exogenous ^{12}C -arginine would not displace either glutamic acid or arginine already adsorbed on the "A" sites and hence would not compete with radioactive pool materials.

Nucleic acids are probably not the R-groups. From the known molar distribution of pool and nucleic acid carbon there would be 3.3 times too many amino acids for a one-to-one correlation between pool amino acid and nucleic acid nucleotide residues.

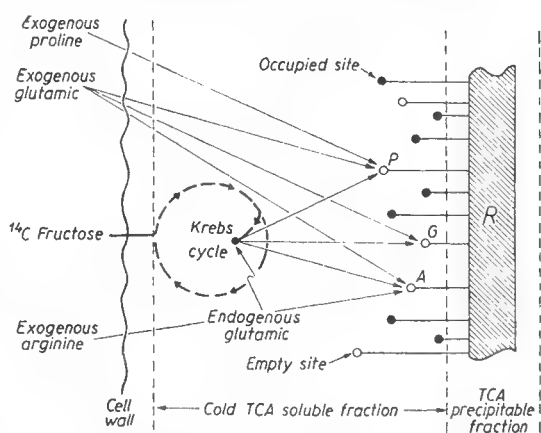


Fig. 13. Model to explain behavior of glutamic family of amino acids.

The only single cellular component present in sufficiently large quantity is the protein. A mixture of protein and nucleic acid cannot be excluded however.

Considerable efforts were made to find peptide linkage among the amino acids in the cold TCA soluble fraction. No peptides were observed and accordingly protein synthesis in *T. utilis*, like *E. coli*⁹, appears to proceed by some process wherein all of the peptide bonds linking together the amino acids into a protein are formed in one event.

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SUMMARY

A study of the kinetics of formation and utilization of metabolic pools of amino acids and phosphorus in *T. utilis* has been carried out using ¹⁴C-fructose and ³²PO₄---. The distribution of these tracers among the chemical fractions of the cell during steady-state conditions was first established. The interrelationships among these fractions in the formation of metabolic pools and in the transfer of pool material for protein and nucleic acid synthesis were then determined. The pools were demonstrated to be complex and so the kinetics of formation and transfer of individual pool amino acids were investigated. These investigations and a study of the effects of exogenous amino acid competitors upon pool amino acids led us to the following conclusions.

1. The carbon of the cold TCA-soluble fraction is mostly contained in amino acids and acts as a metabolic pool for the synthesis of protein and nucleic acid. The TCA-soluble fraction also contains a metabolic pool of phosphorus which supplies phosphorus for nucleic acid and other end products found in the hot TCA-soluble fraction.

2. The results of kinetic investigations permit the distinction between endogenous metabolic pools as essential intermediates for synthesis and reservoir pools not on the main line of synthetic events. The amino acid and phosphorus pools are in the direct line of synthetic events converting exogenous substrate to endogenous macromolecular compounds.

3. The pool amino acids are not free amino acids but are adsorbed to larger molecules and these adsorption sites are intimately connected with the process of protein synthesis.

4. Internal conversion of one pool amino acid to another pool amino acid may occur on a single site prior to incorporation into protein.

5. Nucleic acids are probably not the macromolecules furnishing the adsorption sites since there are more than 3 times as many pool amino acids as there are nucleotides. The protein fraction is the only cellular component present in sufficiently large quantities to furnish the adsorption sites for the pool amino acids. A mixture of nucleic acid and protein is not excluded however.

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II.D.2 The Use of Metabolic Pools of Purine Compounds for Nucleic Acid Synthesis in Yeast

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INTRODUCTION

The flow of exogenous carbon through a metabolic pool of amino acids has been shown to be a necessary step in the formation of protein in *Candida utilis*¹. Similar kinetic investigations were carried out to ascertain whether pool formation is also essential for nucleic acid synthesis. The results reported here show that purines are accumulated into two chemically and functionally distinct metabolic pools prior to nucleic acid incorporation. Some of the characteristics and interrelationships of these two pools are described and their significance for macromolecule synthesis discussed.

PROCEDURES

Previously described methods¹ were used to culture *Candida utilis* (ATCC No. 9950) in C medium*. The ¹⁴C- or ³²P-labeled compounds used as supplements were prepared from radioactive *Chlorella* or *Escherichia coli*³. For the most part the kinetic studies were carried out with the membrane filter technique described by BRITTEN, ROBERTS AND FRENCH². Where further chemical fractionation was necessary this was done by a modification³ of the SCHNEIDER method⁴. Chromatographic identification of the purine compounds in *C. utilis* was carried out on two-dimensional paper chromatograms. The solvent pairs used for acid hydrolysates of nucleotides and nucleic acids were *tert*.-butyl alcohol/hydrochloric acid and *sec*.-butyl alcohol/water/formic acid (see ref.³, p. 41) and for components of the cold TCA extracts were ethanol/ammonium acetate⁵ (pH 3.8) and ammonium sulfate/isopropanol⁶.

EXPERIMENTAL RESULTS

Pool formation with ¹⁴C-adenine

Candida utilis grown exponentially in C medium which contained nonradioactive fructose incorporated radioactivity from ¹⁴C-adenine. Fig. 1 shows the time course of incorporation of ¹⁴C-adenine carbon obtained in experiments using the membrane filter technique².

Initially the rate of incorporation of ¹⁴C into the cold TCA-soluble fraction was more rapid than incorporation into the fraction containing the nucleic acids (TCA-precipitable fraction) which, after a slight lag, took up the adenine carbon at a constant rate until the supply in the medium approached exhaustion. Eventually all of the incorporated ¹⁴C appeared in the TCA-precipitable fraction of the cell. These results suggest that the exogenous adenine carbon is first incorporated into a cold TCA-soluble pool prior to incorporation into the nucleic acid fraction of the cells.

Transfer of pool adenine to nucleic acid

Immersion of the yeast for 8 minutes in C medium containing trace amounts of high specific radioactivity ¹⁴C-adenine-produced cells with more than 60% of the radioactivity in the cold TCA-soluble fraction. After washing and resuspending such a

* C medium: 2 g NH₄Cl, 6 g Na₂HPO₄, 3 g KH₂PO₄, 3 g NaCl, 0.01 g Mg as MgCl₂, 0.026 g S as Na₂SO₄, 100 ml 10% fructose and 900 ml of distilled H₂O.

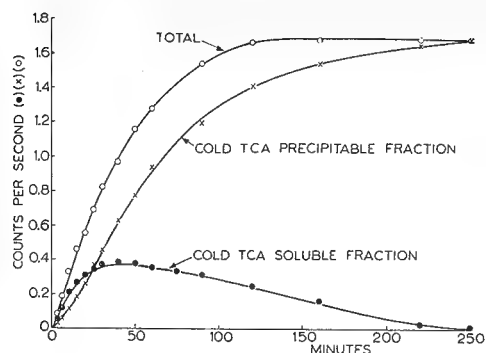


Fig. 1. Time course of incorporation of trace quantities of ^{14}C -adenine by *Candida utilis*. Initial concentration of 0.18 μM adenine per ml medium.

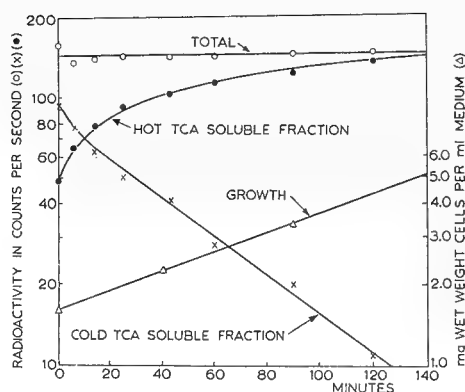


Fig. 2. Kinetics of transfer of pool carbon to nucleic acid during exponential growth.

culture in nonradioactive culture medium it was found that the labeled material rapidly left the cold TCA-soluble fraction, and equally rapidly appeared in the hot TCA-soluble (nucleic acid) fraction (Fig. 2). Little, if any, of the incorporated carbon was lost from the cells during this transformation. Paper chromatography of nucleic acid hydrolysates of the prelabeled cells demonstrated ^{14}C -adenine as the principal labeled component. It may be concluded that the formation of nucleic acid adenine occurs via a metabolic pool of adenine carbon.

Expansion of adenine pool size

When trace amounts of ^{14}C -adenine are used to supplement the medium, there is insufficient exogenous adenine to supply the adenine needs of the growing cell. Consequently, endogenous carbon derived from the ^{12}C -fructose furnishes most of the carbon necessary for nucleic acid adenine synthesis. Studies were therefore made of the incorporation of ^{14}C -adenine carbon supplied at higher exogenous concentrations.

A series of kinetic experiments (*cf.* Fig. 1) yielded data in which the maximum value of adenine pool size could be related to the initial adenine content of the medium. This relationship is shown in Fig. 3 (solid line). Since the cells accumulate adenine avidly, the exogenous concentration continually changes and a steady-state condition cannot be reached over the range of concentration extending to the limit of solubility of adenine in the culture medium. Accordingly, a pool size for a steady-state condition cannot be assigned and no upper limit to the amount of adenine accumulated in the pool could be demonstrated. The maximum observed was 156 μM per g dry cells, calculated as adenine, a value larger than the adenine content of all the nucleic acid in the cell.

Conversion of adenine carbon to guanine

KERR, SERAIDARIAN, AND BROWN⁷ have shown that exogenous adenine is converted by *C. utilis* to adenine and guanine of the RNA. In the present kinetic studies chromatograms of nucleic acid hydrolysates of yeast grown in the presence of ^{14}C -adenine also showed radioactivity to be present in both adenine and guanine. However, the

rate of appearance of radioguanine in the nucleic acids was initially less than for radioadenine. Table I shows the time course of appearance of ^{14}C from ^{14}C -adenine into nucleic acid guanine. The results of chromatographic analysis of hydrolysates of the cold TCA-soluble fraction paralleled those for the nucleic acid fraction. It may be concluded that exogenous adenine is taken into the TCA-soluble pool and in part converted to guanine; both bases are subsequently incorporated into the nucleic acid.

TABLE I
CONVERSION OF ^{14}C -ADENINE* TO ^{14}C -GUANINE BY EXPONENTIALLY GROWING *Candida utilis*

Time in minutes	mg wet wt cells/ml medium	Total radioactivity in hot TCA-soluble fraction (counts per second)	Per cent of total radioactivity of hot TCA-soluble fraction found as guanine
10	1.27	15	26.3
30	1.35	22	34.5
100	1.76	150	39.6
150	2.37	310	44.0
240	3.40	710	45.0

* Initial concentration of ^{14}C -adenine was $0.27\ \mu\text{M}$ per ml medium.

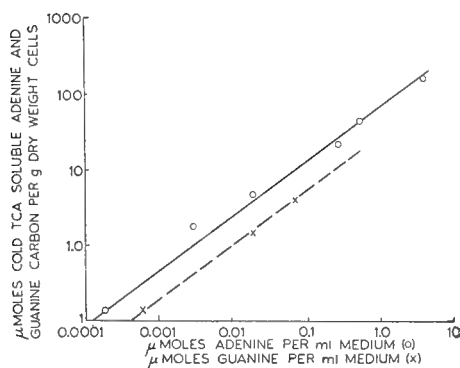


Fig. 3. Expansion of the size of purine pools. The data shown represent the maximum quantity of ^{14}C -adenine carbon incorporated into the cold TCA-soluble fraction as a function of the initial exogenous concentration of ^{14}C -adenine (solid line). Calculations assume all the incorporated radiocarbon remained adenine. Similar data are shown for the expansion of the guanine pool with increasing concentrations of exogenous ^{14}C -guanine (dotted line).

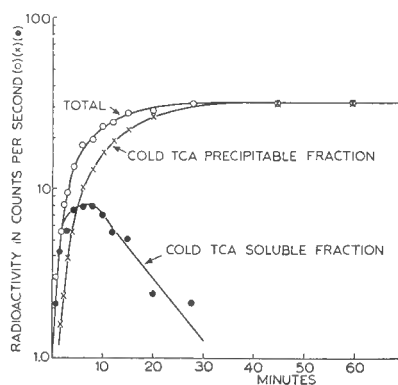


Fig. 4. Time course of incorporation of trace quantities of ^{14}C -guanine during exponential growth. Initial concentration of $0.5\ \mu\text{M}$ moles guanine per ml medium and $4.0\ \text{mg}$ wet weight cells.

Pool formation with ^{14}C -guanine

The results of kinetic experiments with ^{14}C -guanine are generally similar to those described above for the ^{14}C -adenine. Figs. 4 and 5 show the course of uptake of radiocarbon from ^{14}C -guanine. The uptake of ^{14}C -guanine is rapid, ^{14}C appearing first in the TCA-soluble fraction. The TCA-soluble fraction gains in radioactivity until the exogenous supply of ^{14}C -guanine approaches exhaustion. This fraction then loses its radioactivity to the TCA-precipitable fraction, which continues to gain ^{14}C until the TCA-soluble pool is depleted.

The maximum size of the guanine pool is proportional to the amount of guanine initially present in the medium, as observed for the case of adenine supplementation.

The data describing this observation are given in Fig. 3. The largest value observed was $4 \mu M$ per g dry cells. Guanine is much less soluble than adenine and supplements exceeding its solubility in the medium were not used.

Chromatographic analysis of hydrolysates of the cold TCA-soluble and -precipitable fractions showed guanine as the sole radioactive constituent regardless of the external guanine concentration used. Guanine is not readily converted to adenine by exponentially growing *C. utilis*.

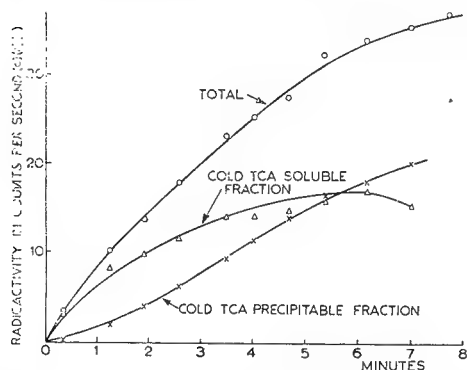


Fig. 5. Short-term experiment showing early kinetics of incorporation of trace quantities of ^{14}C -guanine. Initial guanine concentration of $0.9 \text{ m}\mu\text{moles per ml medium}$ and $3.5 \text{ mg wet weight cells}$.

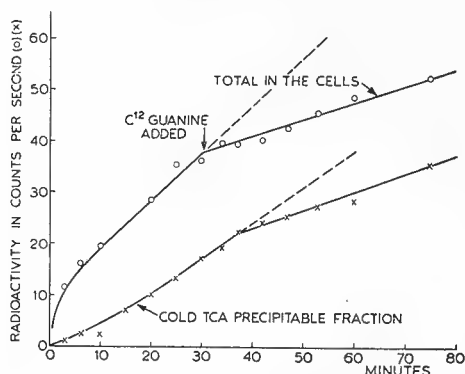


Fig. 6. Kinetics of ^{14}C -adenine incorporation in the presence of ^{12}C -guanine. At $t = 30 \text{ minutes}$ the exogenous concentration of ^{14}C -adenine and ^{12}C -guanine was 0.016 and $0.018 \mu\text{moles per ml medium}$ respectively. Initial cell concentration was $0.5 \text{ mg wet weight cells per ml medium}$.

Isotopic competition results

Additional support for the conclusion that pool adenine is converted in part to pool guanine and that guanine is not readily converted to adenine is given by the isotopic competition results shown in Fig. 6. Fig. 6 shows the results of adding ^{12}C -guanine to a yeast culture growing in a medium containing ^{14}C -adenine. At the time of addition of the guanine, the concentration of the exogenous ^{14}C -adenine was approximately equal to the added guanine. Guanine immediately suppresses ^{14}C -adenine uptake by the cell. Initially the effect is largely upon the uptake into the TCA-soluble fraction. After about 10 minutes the rate of incorporation of ^{14}C into the TCA-precipitable fraction also decreases. On the other hand, there is no effect of ^{12}C -adenine upon the uptake and utilization of ^{14}C -guanine.

The relationship between the phosphorus pool and the purine pool

The kinetics of formation of a pool of phosphorus compounds in *C. utilis* and its utilization for nucleic acid synthesis has been previously described¹. This pool contains one-third of the phosphorus of the cell. Chromatograms (ethanol/acetate: sulfate/isopropanol system) of the cold TCA-soluble fraction from yeast cells grown in C medium and radiophosphorus were very similar to those from *E. coli*⁸. In each case 50% of the phosphorus is found as orthophosphate and most of the remainder is distributed among compounds having R_F 's corresponding to those observed for the nucleoside mono-, di-, and triphosphates of adenine, guanine, cytosine and uracil.

Since the purine pool of the yeast could be enormously expanded upon the addition of exogenous adenine or guanine (Fig. 3), experiments were performed to

ascertain whether a corresponding increase in the phosphorus of the pool also occurred. Fig. 7 shows that the rate of incorporation of radiophosphorus was not altered by the addition of large amounts of adenine. Chromatograms (ethanol/acetate: sulfate/isopropanol system) of the cold TCA-soluble fraction obtained from cells removed just prior to and also 30 minutes after the addition of the adenine, showed no significant differences in the distribution of the radiophosphorus. No free adenine or guanine was observed on the chromatogram of the early sample; the later sample however contained a large quantity of free adenine which was readily detected by ultraviolet light absorption. Free guanine could not be detected. No increase in the amount of purine nucleotide was found. It is concluded that the adenine in the expanded pool was present as the purine base, and not as the nucleotide.

Thus, the yeast cell can contain two kinds of purine pools: a purine nucleotide pool and a pool of purine bases.

The nucleotide pool may be specifically labeled by adding trace quantities of ^{14}C -adenine to the culture medium. Under this condition the ^{14}C -adenine becomes a part of the nucleoside phosphates of adenine and guanine and no free base can be detected. The kinetics of transfer of ^{14}C to nucleic acid from such a labeled nucleotide pool in the presence and absence of nonradioactive exogenous adenine or guanine is shown in Fig. 8. Even though the exogenous purine is accumulated within the cell (*cf.* Figs. 3 and 6), during the course of the experiment its presence as the free base has no effect upon the rate of transfer from the nucleotide pool to the nucleic acid fraction. Thus, rapid mixing of the two pools does not occur. On the other hand the purine base can compete with fructose carbon providing material for the formation

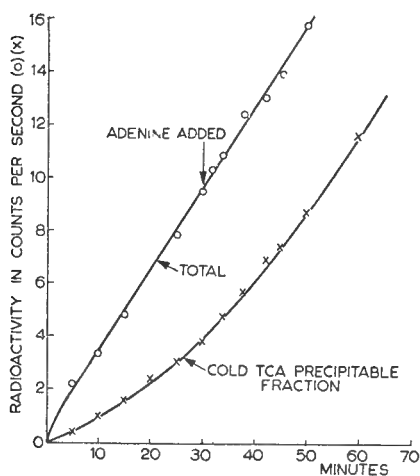


Fig. 7. Kinetics of incorporation of radiophosphorus (^{32}P -orthophosphate) during expansion of adenine pool. ^{12}C -adenine added at a concentration of $1\ \mu\text{mole}$ per ml medium at time indicated and cell concentration was $1.0\ \text{mg}$ wet weight cells per ml medium.

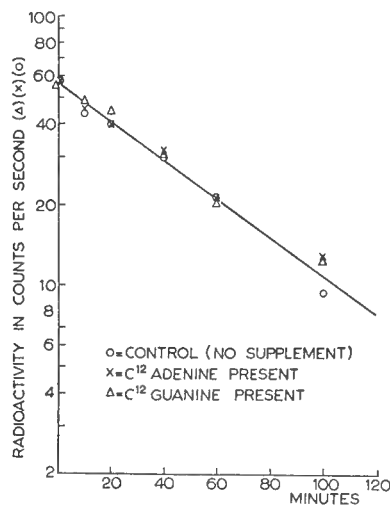


Fig. 8. Loss of ^{14}C -adenine carbon from the cold TCA-soluble fraction. Data obtained from cells briefly immersed in medium containing ^{14}C -adenine (carrier-free) and transferred after washing to nonradioactive medium containing supplements (\times) of ^{12}C -adenine ($0.74\ \mu\text{mole}$ per ml medium); (\triangle) ^{12}C -guanine ($0.005\ \text{mg}$ per ml medium); and (\circ) control (unsupplemented C medium). At $t = 0$, 80% of the incorporated radiocarbon was in the cold TCA-soluble fraction and at the end of the experiment the majority of the radiocarbon was contained in the hot TCA-soluble fraction. There was little loss of pool radiocarbon to the medium.

of the nucleotide; carbon of either source must flow through the nucleotide pool in order to be used for nucleic acid synthesis. Thus, the nucleotide pool serves as a preferred source of carbon for nucleic acid synthesis. From these considerations it is evident that at least two distinct processes are involved in the incorporation of purines into the nucleic acid of yeast. One involves the accumulation of purines within the cell and the other involves alteration and selection for furnishing the proper building blocks for the nucleic acids.

Tests with other nucleic acid compounds

When mixtures of the 3'- and 5'-isomers of ^{14}C -cytidylic or ^{32}P - or ^{14}C -uridylic acids were used as supplements no radioactivity was incorporated by the cells. No diminution was observed in the exponential growth rate upon the addition of the supplements. The specific radioactivity of these compounds was sufficiently high so that trace levels of incorporation should have been observed were these compounds usable or degraded to usable products. In each case there was no detectable radioactivity in the cells even after several generations of cellular growth in the radioactive medium. Labeled nucleosides were not tested.

DISCUSSION

Fig. 9 summarizes the findings presented above in terms of the flow of purine carbon for synthesis of nucleic acid. Two metabolic pools of purine compounds serve as precursor material for nucleic acid synthesis.

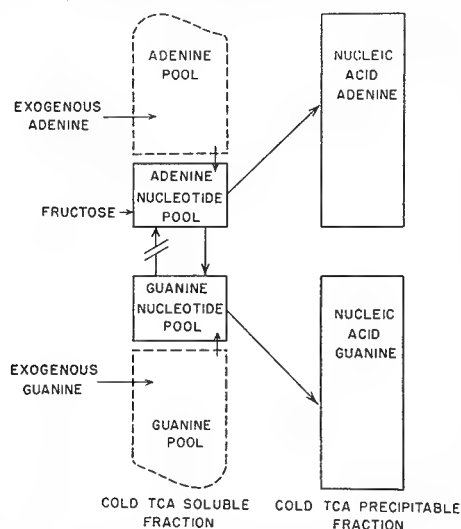


Fig. 9. Flow of purine carbon through metabolic pools in the synthesis of nucleic acid.

radioactive nucleotide pool material, whether the latter is derived solely from fructose or also from exogenous purines.

Pool of purine bases

When the synthetic medium is supplemented by high concentrations of purine bases these materials are found concentrated within the cell at levels exceeding their external concentration. The quantity concentrated is dependent upon the exogenous concentration (Fig. 3). In the case of adenine supplementation the largest pool size

Pool of purine nucleotides

In the absence of purine supplementation a pool of purine nucleotides is formed deriving its carbon solely from the metabolism of fructose. In steady-state cells this pool remains fixed in size and comprises one or two per cent of the total carbon of the cell. It is in this pool that the conversion of one nucleotide to another occurs providing the proper materials for nucleic acid formation. The nucleotide pool is in the direct line of nucleic acid synthesis. The exponential rate of loss of ^{14}C from this pool (Fig. 8) and its subsequent appearance in nucleic acid indicates that there is complete mixing of the radioactive and non-

observed was $57 \mu M$ per ml wet cells ($156 \mu M$ per g dry weight) which is 7.5 times the reported solubility of adenine in water at $25^\circ C$. In the case of guanine supplementation the largest pool size found was $1 \mu M$ per ml of cells or about 30 times the solubility of guanine in water.

The expandable pool may compete with fructose in providing carbon for the nucleotide pool. At the highest internal concentrations almost all of the nucleotide carbon, and consequently nucleic acid purine carbon is derived from this pool rather than the *de novo* synthesis from fructose. On the other hand trace levels of exogenous purines are quickly incorporated into the nucleotide pool without much alteration of the flow of fructose carbon.

The material concentrated in the expandable pool is not converted to other related metabolic products. For example, adenine is concentrated as adenine and remains so until assimilated into the nucleotide pool. Once incorporated into the latter pool, conversion to the guanine nucleotide is possible.

Both pools are extractable with cold TCA, alcohol, or boiling water. Energy is required for pool formation and for transfer of carbon from the expandable pool to the nucleotide pool as well as for ultimate incorporation into nucleic acid. Little or no exchange is observed in the nucleotide pool with exogenous purines. The material concentrated in the expandable pool however has been observed to be less tightly bound. Some exchange with exogenous purines occurs when the pool is greatly expanded.

The mechanism for concentrating purines in the expandable pool is not known nor is the process of maintaining these high concentrations within the cell understood. The fact that purines are held within the cell at levels exceeding their solubility in water suggests that some association of the purine with other cytoplasmic molecules exists.

It is concluded that at least two processes are involved in the incorporation of exogenous purine into nucleic acid. One is the incorporating mechanism that builds up materials to concentrations exceeding that of the external environment. The other is the process in which alteration and selection occur, furnishing the proper compounds for the macromolecules.

SUMMARY

Exogenous purines are incorporated first into metabolic pools and thence into nucleic acids by exponentially growing *Candida utilis*. Kinetic studies show that a purine pool, whose size is determined by the amount of purine in the medium, forms first. This pool contributes material to a nucleotide pool whose pool size is independent of the purine concentration in the medium. Conversion of adenine to guanine occurs in the nucleotide pool which serves as a preferred source of purine for nucleic acid synthesis.

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II.D.3 Metabolic Pools and the Synthesis of Macromolecules

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SUMMARY

Two functionally distinct amino acid pools are found in *Candida utilis*: a *concentrating pool* and a *conversion pool*. The former accumulates exogenous amino acids within the cell to levels exceeding their concentration in the external medium. This pool is only evident when exogenous amino acids are present. Its size is variable and the accumulated amino acids are sensitive to osmotic shock as well as being readily exchangeable with external amino acids. Once concentrated these amino acids may provide material for the conversion pool. Here amino acid interconversion occurs thereby furnishing the amino acids required for protein synthesis. In the absence of exogenous amino acids this pool is formed solely from the carbon and energy source (glucose, fructose, etc.). During exponential growth this pool is always present at a fixed size. This amino acid *conversion pool* is insensitive to osmotic shock. Its content do not exchange with the amino acids of the concentrating pool or with exogenous amino acids.

The results are similar to those obtained in the synthesis of nucleic acids in *Candida utilis* where two *functionally different* and *chemically distinct* pools have been demonstrated. These kinetic investigations describing the flow of exogenous carbon through metabolic pools provide information on some of the preliminary steps in macromolecule formation.

INTRODUCTION

In *Candida utilis* the synthesis of macromolecules from fructose has been shown to occur *via* metabolic pools of amino acids and nucleotides. These pools are a necessary step in the main line of synthesis of protein and nucleic acid. Here conversion of an amino acid or base to others occurs prior to incorporation into macromolecules.

For the synthesis of nucleic acid it was demonstrated that two *chemically distinct* and *functionally different* pools are present¹. The first, a concentrating pool, accumulates nucleic acid *bases* within the cell to levels exceeding the external concentrations. This pool is only evident when the synthetic medium is supplemented with bases. Its size is variable and dependent upon the external concentration of the

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exogenous supplements. Once concentrated, these bases may provide material for the second, a *nucleotide* pool, which is always present, remains constant in size, and, in the absence of exogenous supplements, is derived solely from fructose (or other sugars). Here conversion of one nucleotide to another occurs furnishing the appropriate molecules for nucleic acid synthesis.

It is possible to distinguish two amino acid pools analogous to those used in the synthesis of nucleic acid. The two amino acid pools are defined as follows:

(1) The *internal pool* includes the cold trichloroacetic acid (TCA)-soluble amino acids found in growing cells when glucose, fructose, etc. are the sole carbon source.

(2) The *expandable pool* refers to the additional amino acids which accumulate when the medium is supplemented by amino acids. It was previously shown² that the *internal pool* contains roughly 13 % of the cellular carbon and that the addition of exogenous amino acids produced no immediate exchange with or dilution of the internal pool. This paper reports the results of experiments dealing with the *expandable pool* and with the properties which distinguish the two pools.

PROCEDURES

Previously described methods² were used to culture *Candida utilis* (ATCC No. 9950) in C medium*. Exponentially growing cells were used in all experiments. The ¹⁴C-labeled compounds used as supplements were prepared from radioactive *Chlorella*³. Some of the kinetic studies were carried out with the membrane-filter technique described by BRITTEN, ROBERTS AND FRENCH⁴. Where further chemical fractionation was necessary, this was done by a modification of the SCHNEIDER method⁵. Chromatographic identification of amino acids in the chemical fractions of the cells was carried out on two-dimensional paper chromatograms (see ref. ³, p. 36).

EXPERIMENTAL RESULTS

Formation of expandable pools

Exogenous amino acids are quickly incorporated by exponentially growing cells. Fig. 1 shows the time course of incorporation of trace quantities of ¹⁴C-glutamic acid. The tracer "pulse" appears first in the cold TCA-soluble fraction, and later the transfer of pool radiocarbon (cold TCA-soluble fraction) to the protein fraction is observed. Similar results have been obtained with ¹⁴C-labeled arginine, proline, threonine, valine, ³⁵S-labeled methionine, and ¹⁴C-labeled protein hydrolysates supplied to *C. utilis* growing exponentially in C medium.

When the concentrations of exogenous amino acids are higher, the quantity of amino acids contained in the cold TCA-soluble fraction increases. Table I shows the ¹⁴C-content of the cold TCA-soluble fraction for various concentrations of exogenous ¹⁴C-threonine. Other exogenous amino acids are also concentrated in the cold TCA-soluble fraction as shown in Table II. For comparison, Table III shows the steady-state distribution of pool amino acid and protein amino acid found in cells grown without supplements.

Chromatographic examination of hydrolysates of the cold TCA-soluble and precipitable fractions of cells grown at the highest concentration of exogenous

* C medium: 1 g NH₄Cl, 6 g Na₂HPO₄, 3 g KH₂PO₄, 3 g NaCl, 0.010 g Mg as MgCl₂, 0.026 g S as Na₂SO₄, 100 ml 10 % fructose, and 900 ml distilled H₂O.

threonine (50 μ moles/ml medium) showed that more than 90 % of the radiocarbon in the cold TCA-soluble fraction was contained in threonine; with the remainder in isoleucine. In the protein fraction, however, the ratio of radioactivity in these two amino acids was about one-to-one.

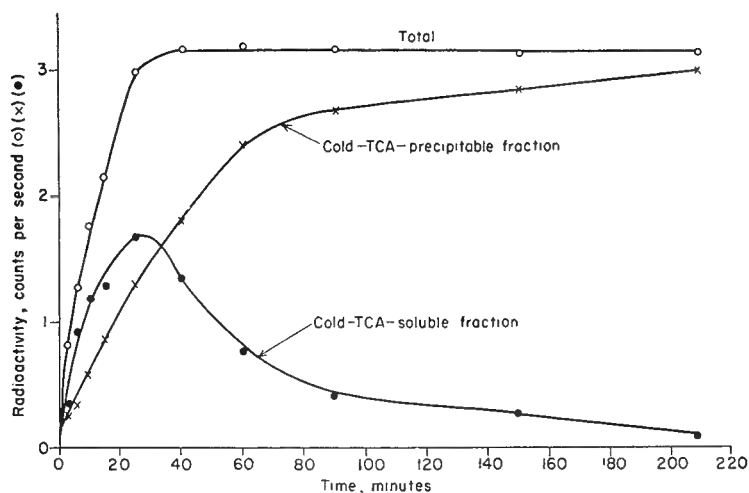


Fig. 1. Time course of incorporation of tracer quantities of ^{14}C -glutamic acid. At time = 0, the exogenous concentration was 0.002 mg glutamic acid per ml medium.

TABLE I
DISTRIBUTION OF RADIOCARBON AMONG POOL AND PROTEIN AMINO ACIDS

Concentration of exogenous ^{14}C -threonine (μ moles/ml medium)	μ moles ^{14}C -threonine per g dry weight cells*	
	Cold TCA-soluble fraction	Cold TCA-precipitable fraction
50.0	442	520
8.4	354	446
6.7	268	328
5.0	190	272
3.4	104	149
1.7	32	82
0.8	9	41

Cells grown from light inoculum to about 2.3 mg wet weight of cells per ml medium.

* Calculated on the basis that all the radiocarbon incorporated remained ^{14}C -threonine.

TABLE II
AMINO ACID ACCUMULATION OF THE COLD TCA-SOLUBLE FRACTION

Experiment	Exogenous ^{14}C amino acid	Concentration (μ moles/ml medium)	Cold TCA-soluble fraction (μ moles/ g dry weight cells)
1	Arginine	1.1	158
2	Proline	1.7	170
3	Valine	1.7	225

In each experiment, cells grown exponentially for 150 min in C medium containing the labeled amino acid.

TABLE III

STEADY-STATE DISTRIBUTION OF RADIOCARBON AMONG POOL AND PROTEIN AMINO ACIDS*

Component	Pool quantity of compound $\mu\text{moles/g dry}$	Protein quantity of compound $\mu\text{moles/g dry}$
Isoleucine-leucine	117	785
Lysine	24	625
Glutamic acid	290	640
Aspartic acid	9	762
Valine	65	512
Alanine	240	695
Threonine	8	455
Serine	8	600
Proline	7	287
Arginine	63	210
Glycine	108	488
Per cent accounted for	87	85

* Data obtained from cells growing exponentially in C medium containing ^{14}C -fructose.*Properties of the expandable pool*

The amino acids of the internal pool are not lost to the medium during exponential growth, nor are they exchanged with exogenous amino acids². In the expandable pool, however, the situation is quite different, shown by the following type of experiment. Cells were grown in C medium supplemented by a high concentration of ^{14}C -arginine (1.0 mg/ml medium).

The kinetics of incorporation of radiocarbon into pool and protein followed during this labeling growth period showed that approximately $\frac{2}{3}$ of the protein arginine was being derived from fructose carbon and $\frac{1}{3}$ from the amino acid supplement. After $3\frac{1}{2}$ h of growth the cells were harvested, washed twice with C medium and an aliquot transferred to C medium containing nonradioactive arginine (1.0 mg/ml) on the one hand (A), and no supplement on the other (B), Fig. 2. At the time of transfer, 57 % of the incorporated radiocarbon was contained in the cold TCA-soluble fraction. Optical density measurements indicated that growth proceeded after the transfer with no delay in the unsupplemented culture (B), and with a slight delay (10 min) in the other (A).

Both groups of cells lose radiocarbon to the medium, as is shown in Fig. 2, but the loss in (B) (no supplement) is less rapid than in (A). In (B) reincorporation of the undiluted radioactivity becomes evident in about 1 h. These results may be interpreted as follows. Where no supplement was added (B), the expandable pool decreases for two reasons: there is a continuous flow into the internal pool and thence into protein; and there is an approach to a new equilibrium of the expandable pool with the medium. When the expandable pool has reached a small enough value through these two processes, and the external concentration has reached a large enough value, reincorporation of radiocarbon occurs. In experiment (A), the loss of expandable pool radiocarbon is due to transfer through the internal pool into protein, and to exchange with the nonradioactive arginine in the medium. This exchange process is faster than the loss of pool material in (B). The continuous and large dilution in experiment (A)

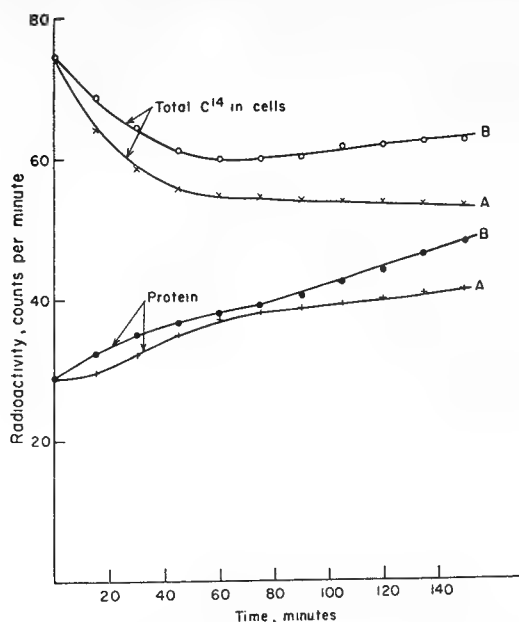


Fig. 2. Upper curves = loss of radioarginine from cells after transfer to nonradioactive C medium containing 1.0 mg ^{12}C arginine/ml (A) or unsupplemented C medium (B). Lower curves = incorporation of radioarginine carbon into protein fraction of *C. utilis* after transfer of labeled cells to nonradioactive C medium. (A and B as above).

results in a steadily decreasing specific radioactivity of both amino acid pools. Consequently, there is a steadily decreasing rate of radiocarbon incorporation into the protein. The protein-incorporation curve can be quantitatively predicted on the assumption that the cell processes as measured in the $3\frac{1}{2}$ h prelabeling period continued (except for the slight lag) and the only change of circumstances was the introduction of the exchange of radioactivity between the pool and medium.

In the case of the unsupplemented medium (B), the rate of incorporation of radiocarbon into protein is not immediately changed by the transfer to the non-radioactive medium, but this rate falls as radiocarbon from the expandable pool is lost to the medium. Apparently the proportion of arginine carbon derived from exogenous sources is a function of the size of the expandable pool. As the flow from the expandable pool decreases, the specific radioactivity of the internal pool also decreases, and there is a consequent reduction in the rate of incorporation of radiocarbon into protein.

In Fig. 3 the logarithms of the radioactivity in the cold TCA-soluble fraction in the two experiments described above are plotted against the time after the transfer to the nonradioactive medium. In both experiments the data can be approximated by a pair of straight lines. In (A) the early, fast component presumably represents the combined effects of exchange with nonradioactive arginine in the medium and transfer to protein *via* the internal pool, whereas the slow component reflects the decrease in specific radioactivity of the internal pool. Since the internal pool is constant in size and undergoes no exchange, the rate of dilution of its specific radioactivity is limited by the rate of protein synthesis. In (B) the slower component again

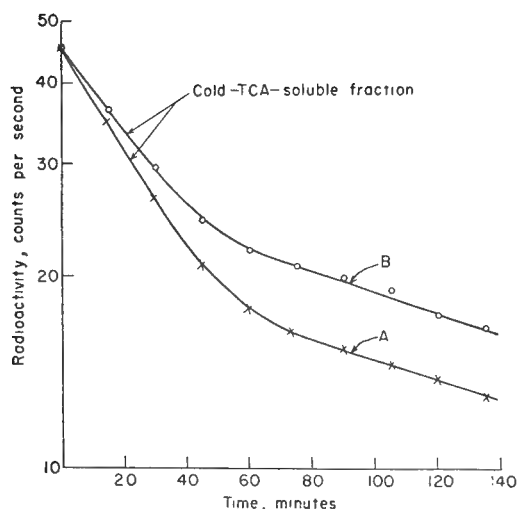


Fig. 3. Loss of pool radioarginine after transfer of labeled cells to nonradioactive C medium A and B as in Fig. 2).

reflects the transfer of internal pool carbon to protein while the faster component includes the approach to the new equilibrium between the expandable pool and the medium.

Effect of hydrostatic pressure

Studies have been made of the effect of pressure treatment on the stability of the amino acid pools in yeast.

Exponentially growing cells were labeled by a 2-min immersion in C medium containing carrier-free ^{14}C -fructose. The cells were washed by centrifugation in non-radioactive C medium and then resuspended in C medium without fructose. These cells contained 87 % of the incorporated radiocarbon in the cold TCA-soluble fraction.

An aliquot of these cells was compressed in a pressure cell⁶ at 30,000 lb./sq. in. This pressure was applied and released 10 times over a 5-min interval. ^{12}C -fructose was added to the treated culture, the cells were aerated at 30°, and the growth and the fate of the labeled pool carbon were measured. Fig. 4 shows that 64 % of the incorporated radiocarbon was lost from the cells to the medium during the first 90 min of aeration. The remaining pool radiocarbon was transferred to protein or nucleic acid fractions. No increase in optical density was observed during the first 100 min; by 120 min the cells were growing at the optimal rate.

A control culture of these labeled cells not subjected to pressure continues to grow exponentially upon addition of fructose and lost only a few per cent of incorporated radiocarbon to the medium during the 90-min interval.

Simple rupture of cellular membranes cannot be responsible for the release of internal pool amino acids to the medium. No loss of the pool is observed after the pressure treatment unless fructose is added to the culture. Thus, both energy and time are required for the release of the pool materials. Furthermore, microscopic examination during the course of the experiment showed no cell fragments or visible cellular alterations.

One explanation of the observed results is that pressure treatment disrupts the

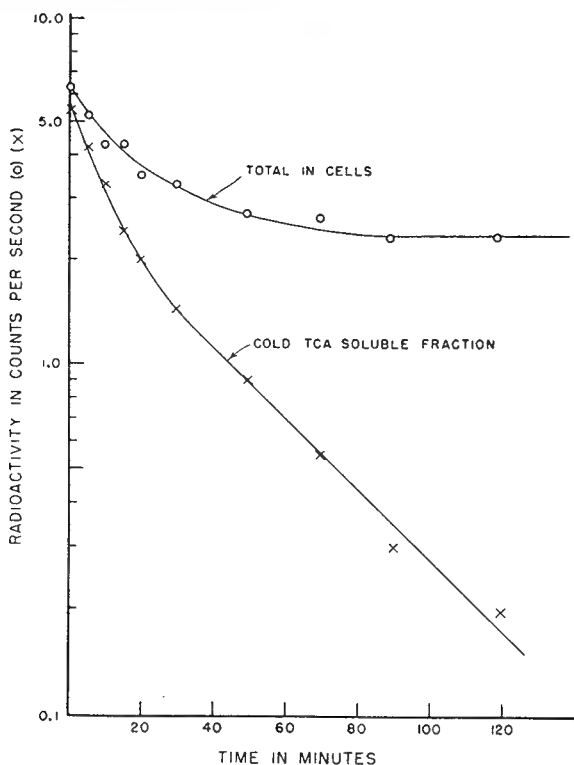


Fig. 4. Loss of radiocarbon from cells (upper curve) and metabolic pools (lower curve) after cells were subjected to a pressure of 30,000 lb./sq. in.

organization, and reorganization must occur before growth resumes. Evidence supporting this conclusion was obtained in another experiment by following the kinetics of ^{14}C -fructose incorporation immediately following the pressure treatment. Fig. 5 shows that rapid incorporation of fructose carbon occurs. Initially this incorporation is due to pool reconstruction, since most of the incorporated radiocarbon appears in the cold TCA-soluble fraction. The pool appears to reach saturation in 90 min at which time the quantity of radiocarbon contained in the cold TCA-soluble fraction corresponded to two thirds of the normal steady-state value of the internal pool. This incorporation is approximately equal to the quantity of pool material lost from the cells (Fig. 4) and therefore represents replenishment of the pool. No increase in the optical density of the cells was observed until 120 min after the addition of the fructose.

The effect of osmotic shock

The internal pool of amino acids is relatively insensitive to osmotic shock. ^{14}C -fructose labeled cells lose from 3 to 8 % of their internal pool material when suspended for half an hour in distilled water. About the same loss is observed when these labeled cells are transferred to nonradioactive C medium. The amino acids in the expandable pool on the other hand, behave quite differently as shown below.

Cells were grown for several hours in medium containing 6 μmoles ^{14}C -threonine per ml, then harvested and washed twice in unsupplemented medium. An aliquot

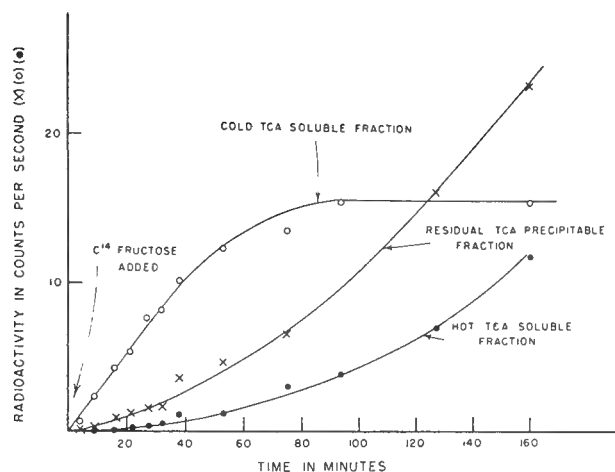


Fig. 5. Kinetics of incorporation of ^{14}C fructose in *C. utilis* after cells were subjected to a pressure of 30,000 lb./sq. in.

was chemically fractionated and the quantity of ^{14}C -threonine carbon found in the cold TCA-soluble fraction corresponded to $56\ \mu\text{moles/g}$ dry weight of cells. This value is seven times the concentration found for threonine in the internal pool (see Table III). When another aliquot of these labeled cells was suspended in water, the cells immediately lost 50 % of the total pool radiocarbon to the water.

The internal pool of amino acids in cells subjected to a pressure of 30,000 lb./sq. in. becomes sensitive to osmotic shock. Cells labeled with a 5-min immersion in medium containing ^{14}C -fructose were harvested and were found to have 80 % of the incorporated carbon in the cold TCA-soluble fraction. An aliquot of these cells after the pressure treatment lost 31 % of the internal pool carbon when suspended in distilled water. A control suspension (no pressure) lost 6 % to the water wash.

DISCUSSION AND CONCLUSIONS

Two functionally distinct amino acid pools exist in *Candida utilis*. The major characteristics of these pools are compared in Table IV.

The fact that these two pools display such different characteristics and do not rapidly equilibrate with each other in the cell indicates that they are physico-

TABLE IV
MAJOR CHARACTERISTICS OF AMINO ACID POOLS IN *Candida utilis*

Characteristic	Expandable pool	Internal pool
Function	Concentrates exogenously supplied amino acids	Interconverts and selects amino acids for protein incorporation
Size	Variable and dependent upon exogenous amino acid concentration	Constant
Stability	Sensitive to osmotic shock. Exchanges with exogenous amino acids	Insensitive to osmotic shock. Not exchangeable with amino acids. (Expandable pool or exogenous)

chemically distinct. It seems unlikely that phosphorylated forms could account for this difference since there is insufficient pool phosphorus (300 μ moles/g dry weight cells) available for the 1000 μ moles of amino acids in the internal pool. The expandable pool can be even larger than the fixed internal pool, but no additional incorporation of ^{32}P occurs when large quantities of exogenous amino acids are rapidly accumulated in the expandable pool. Thus neither the internal nor the expandable pool differences can be explained on the basis of phosphorylation of the amino acids. Some correlation between phosphorus turnover and amino acid accumulation would be expected if phosphorus were associated with these amino acids.

The mechanism concentrating exogenous amino acids in the expandable pool is unknown, and no alteration of the amino acids in the internal pool has been detected. It has been suggested that the internal pool is complexed with macromolecular components of the cell². The only class of substances present in sufficient quantity to accommodate the internal pool amino acids are the proteins and would require that 25 % of the protein be so involved. Nucleic acids do not appear to be the macromolecules forming the amino acid-R-group complex. From the known molar distribution of internal pool and nucleic acid carbon there would be 3.3 times too many amino acids for a one-to-one correlation between pool amino acid and nucleotide residues. A mixture of protein and nucleic acid could, of course, serve as a complexing system.

It seems likely that macromolecules, most probably the proteins, are the sites of association for the internal pool amino acids. These amino acids are on the main line of protein synthesis, and are more tightly bound than those in the expandable pool. The internal pool is more closely linked to the mechanisms of protein incorporation, for it is here that amino acid interconversions and selection for protein incorporation occur. The sensitivity of the internal pool to hydrostatic pressure is probably due to distortion of the structure binding the pool amino acids.

In the expandable pool the amino acids accumulated do not interconvert. The threonine concentrated by the cells grown on the highest exogenous threonine concentration (Table I) remained, for the most part, as pool threonine, but a small portion was converted to pool isoleucine. This isoleucine and a small quantity of the total pool threonine are components of the internal amino acid pool as protein threonine and isoleucine both become about equally radioactive.

Fig. 6 schematically presents an interpretation of the results presented above. When fructose is the sole carbon source, only the internal pool is evident, and all the protein carbon comes from this source. At some point in the synthesis from fructose the amino acid "family heads" are formed and made available to the internal pool. In the internal pool the family heads are converted to their respective family members.

The expandable pool becomes evident when the synthetic medium is supplemented with amino acids. This pool reflects both the quantity and kind of exogenous supplement used. The accumulated materials exceed the external concentration but do not get converted to other amino acids. This concentrated material competes with the amino acids formed from fructose for utilization in the internal pool. This competitive reaction is controlled by a number of variables: the quantity of expandable pool material accumulated, the rate of production from fructose of that amino acid, and the quantity of material required for the internal pool, (which reflects in turn the protein requirements and the metabolic state of the cell).

Once incorporated in the internal pool, further competition of these pool amino

acids with exogenous amino acids, expandable pool material, or with newly synthesized amino acids from fructose does not occur. The competition must occur at the time of incorporation into the internal pool or prior to incorporation into this pool.

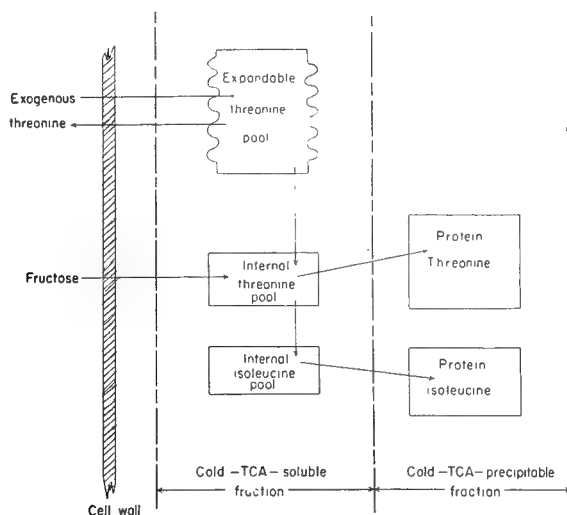


Fig. 6. Flow of threonine carbon through metabolic pools in the synthesis of protein.

These results are similar to those obtained in the synthesis of nucleic acid *via* metabolic pools¹. These kinetic investigations describing the flow of exogenous carbon through metabolic pools and hence into macromolecules provide a more definite picture of some of the preliminary steps in protein and nucleic acid formation. In addition, they reveal that the amino acid and base composition of the cell may vary, reflecting the kind and quantity of exogenous molecules. This altered composition, in turn, affects the endogenous flow of fructose carbon which, in the absence of other exogenous organic molecules is a satisfactory carbon source. Such studies also demonstrate the great capacity of some living cells to utilize ever-changing environments economically.

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II.D.4 Metabolic Pools and the Utilization of Amino Acid Analogs for Protein Synthesis

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SUMMARY

Studies of the kinetics of analog and amino acid incorporation into metabolic pools and proteins of yeast and *E. coli* have shown that at least two processes are involved in the selection of the natural amino acid in preference to the analog. These selection steps occur in the formation of the metabolic pools, the first occurring in the concentrating system of the cell, which accumulates exogenous amino acids or analogs into the cell at levels exceeding external concentrations. In the yeast, the final distinction between analog and amino acid occurs at the time of entry into a second metabolic pool (internal pool). In this pool conversion of amino acids to the required end products occurs. Once incorporated into this pool no further selection occurs; the ratio of analog to amino acid in this pool and in protein is the same.

INTRODUCTION

The demonstration that amino acid analogs could be incorporated into bacterial proteins¹⁻⁹ raised many questions concerning (a) the nature of the protein synthesized and (b) the actual mechanism of analog incorporation. It has been shown that the substitution of the analogs norleucine or selenomethionine for methionine in the proteins of *E. coli* did not result in the synthesis of radically different molecular species, but that the macromolecules formed had physicochemical properties similar to the proteins normally synthesized^{4,10}. Furthermore, each methionine site in the proteins seemed to have the same susceptibility for analog substitution, that is, norleucine¹⁰ (and selenomethionine⁴) replaced methionine *in the same proportion* in all of the proteins examined. Recently it has been shown that the substitution of ethionine for methionine in a single protein (α -amylase) was the same for each individual methionine site¹¹.

As has been pointed out by YOSHIDA AND YAMASAKI¹¹, no evidence has been presented for the mechanism of analog incorporation into protein. It is evident that the bacterial cell possesses certain mechanisms for selecting the natural amino acid and rejecting, at least partially, the amino acid analog. With norleucine a large environmental pressure was required in order to effect a relatively small change in protein composition; a ratio in the medium of norleucine to methionine of 100 resulted

in only a 40 % replacement of methionine by the analog¹⁰. Studies of the kinetics of analog incorporation into the metabolic pools and proteins of yeast and *E. coli* were made to determine where these selections among amino acids and their analogs occurred.

PROCEDURES

Wild type *E. coli* ML 30 and the yeast, *Candida utilis* were used in these experiments. Both types of cells were cultured in vigorously aerated C medium* with maltose as the carbon source for *E. coli* and fructose employed with *Candida utilis*.

DL-[3-¹⁴C]-*p*-fluorophenylalanine was obtained from the Volk Radiochemical Company, Chicago, Illinois (79.2 mg/mC). Uniformly-labeled L-[¹⁴C]phenylalanine was obtained from Nuclear-Chicago Corporation, Chicago, Illinois (16 mg/mC). These materials were always added directly to exponentially-growing cultures of cells with appropriate carrier compound to bring the concentration to the desired levels.

Culture samples of *E. coli* were withdrawn at various times and the cells removed by centrifugation. The cell pellet was washed by resuspending in 40 ml C medium and centrifuged. The resulting pellet was resuspended in 5 ml of 5 % cold trichloroacetic acid (TCA) and an appropriate aliquot measured to determine the total radioactivity taken up by the cells. The remaining suspension was centrifuged and the radioactivity of the TCA-soluble fraction (containing the metabolic pool) and of the TCA-insoluble fraction (containing the proteins) were measured to determine the distribution of radioactivity between pool and protein.

The extracting process used for *Candida utilis* was the same as that given above, except where it was desired to separate the two metabolic pools (see below) found in the yeast¹⁵. After washing in C medium the concentrating pool was extracted by resuspending the pellet of cells in 20 ml cold distilled water for 20 min.

An appropriate aliquot of this suspension was measured for the total radioactivity taken up by the cells. The remaining suspension was centrifuged to separate the water soluble fraction containing the expandable pool material. The pellet was treated with 5 % TCA and the distribution of tracer between the internal pool and protein was determined from measurement of radioactivity in the TCA soluble and insoluble fractions respectively.

RESULTS

E. coli

Kinetics of amino acid analog incorporation: The kinetics of incorporation and utilization of amino acid analogs are in many respects similar to those observed with the natural amino acids¹²⁻¹⁶. For example, at low external concentrations the analogs can be accumulated in the metabolic pool of the cell up to levels exceeding their external concentrations. This accumulation is rapid and precedes the appearance of the analog in the protein. Fig. 1 shows the time course of incorporation of tracer quantities of DL-[3-¹⁴C]-*p*-fluorophenylalanine (*p*FPhe) into the pool and protein**.

* C Medium: 2 g NH₄Cl, 6 g Na₂HPO₄, 3 g KH₂PO₄, 3 g NaCl, 0.01 g Mg as MgCl₂, 0.026 g S as Na₂SO₄, 100 ml 10 % maltose or fructose, and 900 ml distilled H₂O.

** *p*-fluorophenylalanine has been shown by MUNIER AND COHEN⁹ to replace only phenylalanine in the proteins of *E. coli*.

The limited exogenous supply of the analog is soon exhausted and incorporation into protein rapidly depletes the analog in the pool.

At higher external concentrations of analog a proportionally larger quantity of accumulated material is observed in the cells. Fig. 2 (upper curve) shows the quantity of analog contained in the cold TCA-soluble fraction as a function of external concen-

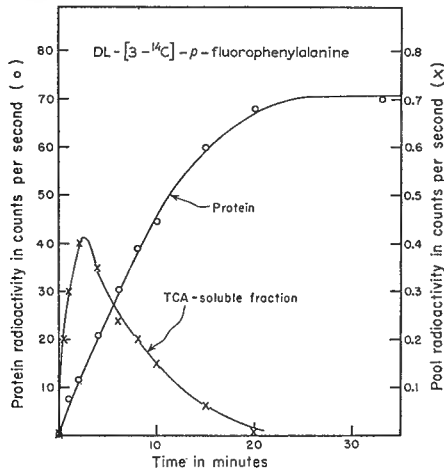


Fig. 1. Kinetics of uptake of tracer quantities of DL-[3-¹⁴C] *p*-fluorophenylalanine (0.002 mg/ml medium) into pool and protein of *E. coli*.

tration. Two processes appear to be involved in the uptake of *p*FPhe. One, a process concentrating analog in the cell in excess of the external concentration, appears to saturate at low concentrations. At exogenous levels exceeding 0.03 mg *p*FPhe/ml medium a second process of incorporation is observed. The quantity of material taken into the pool through this second process is directly proportional to the external concentration (dashed curve, Fig. 2). Pool accumulation to levels exceeding the external concentrations does not occur through this process. Incorporation of the analog into protein, however, as shown in Table I, depends directly upon the total quantity of pool material available, regardless of the mechanisms of uptake.

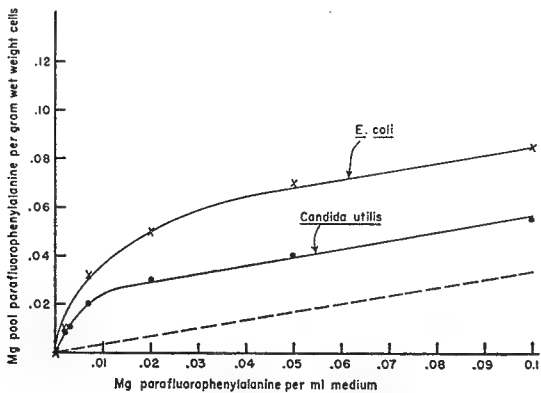


Fig. 2. Maximum level of *p*-fluorophenylalanine pool observed in *E. coli* and *C. utilis* as a function of external concentration of analog. Dashed curve shows component of accumulation which is directly proportional to exogenous concentration.

TABLE I
DISTRIBUTION OF PARAFLUOROPHENYLALANINE IN *Escherichia coli*

Medium mg pFPhe/ml (at $t = 0$)	Pool* mg pFPhe/g wet weight cells	Protein** mg pFPhe/ Δ g wet weight cells	Protein/pool***
0.002	0.008	0.2	25
0.007	0.032	0.8	25
0.02	0.050	1.2	24
0.05	0.068	1.5	22
0.10	0.085	2.2	28

* Maximum level observed.

** Value calculated from steady state rate of incorporation of *p*-fluorophenylalanine per unit quantity of newly formed cells.

*** Δ gram wet weight cells = mass of cells grown after the addition of the tracer material.

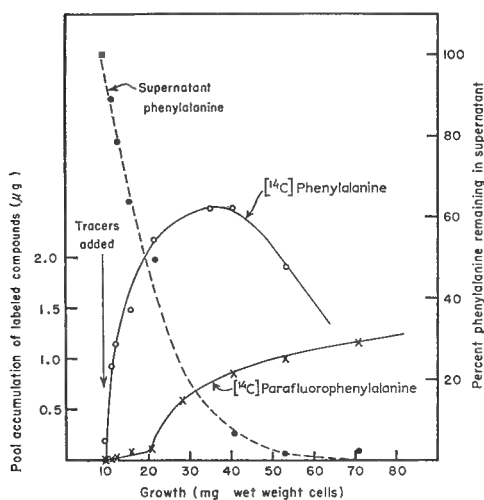


Fig. 3. O, accumulation of L-phenylalanine; \times DL-*p*-fluorophenylalanine in the pool of *E. coli*.

An exponentially growing culture of cells was divided. Culture 1 (O) was supplied with L- $[^{14}\text{C}]$ -phenylalanine and an equimolar quantity of DL- $[^{14}\text{C}]$ *p*-fluorophenylalanine. Culture 2 (\times) was treated identically, but with reciprocal labeling. Initial exogenous concentration of each compound was 0.0067 mg/ml medium. ●, concentration of phenylalanine remaining in medium. Growth of the two cultures was identical.

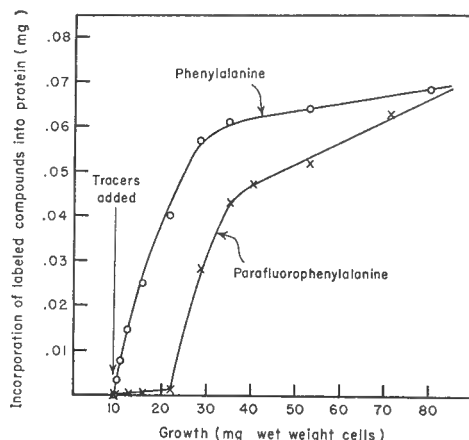


Fig. 4. O, incorporation of L-phenylalanine; \times , incorporation of DL-*p*-fluorophenylalanine into proteins of *E. coli*. Conditions as described in Fig. 3.

Competitive utilization of amino acids and amino acid analogs: Kinetics of analog incorporation were significantly influenced by the presence of natural amino acids in the medium. Figs. 3 and 4 show the kinetics of pool accumulation and protein incorporation of phenylalanine and *p*FPhe. Initially very little of the analog was accumulated in the metabolic pool or incorporated into protein. On the other hand, phenylalanine was immediately concentrated by the cell and utilized for protein synthesis. When phenylalanine accumulation no longer continued at the maximal rate, due to depletion in the supply, accumulation of the analog into the pool occurred. Subsequently, the analog was incorporated into protein.

The analog, however, was not completely excluded from the cell, even during these early stages of phenylalanine incorporation. Fig. 5 shows the relative rates of

protein incorporation of DL-[^{14}C]-*p*-fluorophenylalanine in the absence or presence of equimolar quantities of L-phenylalanine. The rate of protein incorporation of *p*FPhe was reduced by a factor of 100 when phenylalanine was present.

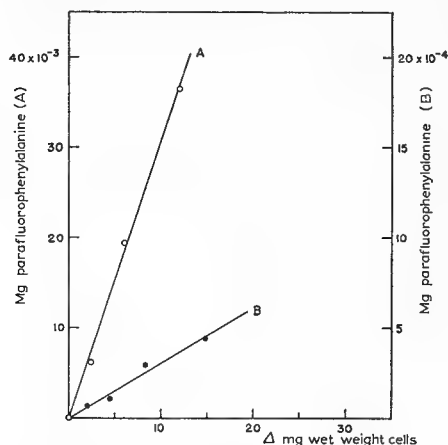


Fig. 5. Protein incorporation by *E. coli* of *p*-fluorophenylalanine. A, supplied in the absence of an equimolar quantity of phenylalanine; B, in the presence of an equimolar quantity of phenylalanine.

Candida utilis

Kinetics of amino acid analog incorporation: Similar experiments have been done with the yeast, *C. utilis*. In this organism *two* functionally distinct metabolic pools of amino acids have been found¹⁵. The first accumulates the amino acids within the cell to levels exceeding their external concentration. This pool has been called the expandable (or concentrating) pool¹⁵ and has many of the characteristics of the amino acid-concentrating system observed in *E. coli*. The size of this pool varies with external concentration and the pool is only evident when amino acids (or analogs) are present in the medium. Material in this pool is sensitive to osmotic shock and readily exchanges with external amino acids.

A second pool, called the internal (or conversion) pool¹⁵ is always found in exponentially-growing cells. The amino acids contained in this pool are on the main line of synthetic events. It is here that the conversion of one amino acid to others occurs, furnishing the appropriate molecules for protein incorporation. Once in this pool, these amino acids do not exchange with exogenous or accumulated amino acids, nor are they sensitive to osmotic shock. In the absence of exogenous amino acids (or analogs), this pool is formed solely from the carbon source (sugar). During exponential growth the size of this pool remains fixed and contains 13 % of the total cellular carbon. The presence of exogenous or accumulated amino acids in the cell does not alter the pool size.

In addition to these functional differences, the two pools can be extracted separately. The expandable pool alone is extractable with cold water; both pools are extractable with cold trichloroacetic acid.

Despite the existence of such amino acid pools, very little *p*FPhe can be *concentrated* by the yeast cells. Fig. 2 shows the total quantity of this analog contained in both pools (TCA-soluble fraction) as a function of exogenous analog concentration. These data are similar to those obtained with *E. coli* (Fig. 2), except that in the

yeast cell saturation of the concentrating system occurs at a lower external level. Very little *p*FPhe is found in the metabolic pools in excess of the external concentration. In the yeast, as in *E. coli*, protein incorporation of the analog was found to be directly dependent upon pool concentration. As a consequence of this reduced capacity for accumulation, analog replacement in the yeast cell requires a higher external concentration than in *E. coli* for the same degree of protein substitution.

Competitive utilization of amino acids and amino acid analogs: In the yeast our principal interest has centered on the internal pool of amino acids, because of its necessary and essential role in the synthesis of protein. Kinetic interrelationships among the internal pool, the expandable pool and the proteins are difficult to measure, however, at low external concentrations of *p*FPhe because of the possibility of saturation of the concentrating system for the analog.

At high concentrations of DL-*p*FPhe and L-phenylalanine ($10^{-3} M$ and $10^{-4} M$ respectively) added simultaneously to a growing culture of *Candida utilis*, both compounds are taken up by the cells in easily measurable quantities. Two identical flasks with reciprocal labeling ($[^{12}C]$ Phe + $[^{14}C]$ *p*FPhe in one and $[^{14}C]$ Phe + $[^{12}C]$ *p*FPhe in the other) were inoculated with cells from an exponentially-growing culture of *C. utilis*. The distribution of the analog and phenylalanine was obtained by following kinetics of incorporation of the two labeled compounds in the expandable pool, the internal pool, and the protein. The results of a typical experiment are shown in Table II.

TABLE II
DISTRIBUTION OF PHENYLALANINE AND *p*-FLUOROPHENYLALANINE IN *Candida utilis*
External ratio of DL-*p*-fluorophenylalanine to L-phenylalanine was 10:1.

	Expandable pool (μ moles/g wet weight cells)	Internal pool (μ moles/g wet weight cells)	Protein* (μ moles/ Δ g wet weight cells)
<i>p</i> FPhe	23.9	14.0	725
Phenylalanine	4.58	6.1	305
Ratio <i>p</i> FPhe/Phe	5.2	2.30	2.38

* Δ gram wet weight cells = mass of cells grown after the addition of the tracer materials.

The yeast cells, given an external molar ratio of analog to amino acid of 10/1, contained these materials in the expandable pool at a ratio of 5.2/1. The ratio of the analog to amino acid in the internal pool was found to be 2.3/1 and was identical to the ratio obtained in the proteins. It appears that no further selection occurs by the processes through which the materials of the internal pool are made into protein.

DISCUSSION

The results obtained from the studies of the kinetics of utilization of *p*FPhe indicate that in yeast at least two processes exist selecting the natural amino acid in preference to the analog for protein synthesis. It is possible to correlate these results with schemes¹⁴⁻¹⁶ describing the flow of endogenously synthesized amino acid carbon, and to show the alterations in the carbon flow produced by exogenous amino acid. Fig. 6 summarizes some of the essential details.

In the absence of exogenous amino acids (or analogs), all of the amino acid requirements of the cell are derived from the sugar carbon and CO_2 . "Families"¹⁷ of amino acids are formed from "parent" members: the parent is synthesized in the amino acid production system (Fig. 6) with the conversion of the parent to related family members occurring in the internal pool^{14,15}.

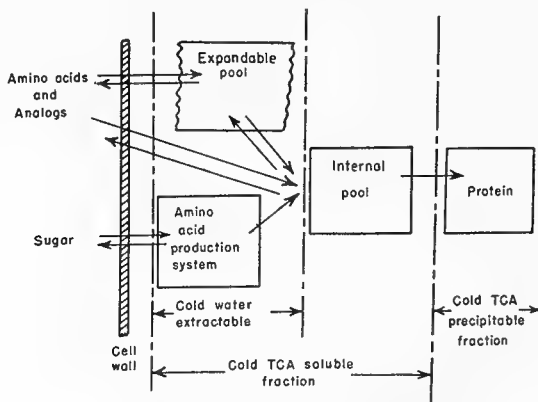


Fig. 6. Carbon flow in *C. utilis*.

The addition of amino acids or analogs to the medium results in their rapid cellular accumulation by a concentrating system (expandable pool) to levels exceeding (a) the external concentration, and (b) the intracellular level usually maintained through endogenous amino acid production from sugar. The accumulated amino acids mix with those amino acids endogenously formed, and from this mixture are withdrawn the molecules required for the internal pool and protein synthesis.

One of the first steps in the selection between an exogenous amino acid and an analog occurs in the concentrating system: With *E. coli*, accumulation of exogenous *p*FPhe is markedly reduced by the addition of equimolar quantities of phenylalanine (Fig. 3). It is evident that phenylalanine has a greater affinity for the concentrating system than *p*FPhe has. With *C. utilis*, this preference for phenylalanine is also observed (expandable pool, Table II), although not to the same extent as measured with *E. coli*.

Another process appears to be involved in the uptake of *p*FPhe, by-passing the concentrating system as shown in Fig. 6. Analog accumulation in the cell in excess of the external concentration does not occur beyond certain levels of exogenous *p*FPhe. However, with increasing external concentrations, larger and larger quantities of *p*FPhe are found in the cold TCA soluble fraction of the cell. After saturation of the concentrating system, the *p*FPhe taken up per unit volume of cells is proportional to, but smaller than, the external level (Fig. 2). Evidence supporting this interpretation is found in the data of HALVORSON AND COHEN¹⁶. These authors suggested that in *Saccharomyces cerevisiae* exogenous amino acids could be used for protein synthesis without equilibrating with the expandable pool.

Thus materials available for utilization in the internal pool are dependent upon a number of environmental and cellular factors. These are: (a) The ability to concentrate exogenous materials. This process depends upon the kind of exogenous supplements available, the relative specific affinity of each substrate for the concentrating mechanism, and the degree to which each substance can be accumulated (pool satu-

ration). (b) An entry process where cellular uptake is directly proportional to the external concentration. (c) Endogenous amino acid production.

Incorporation into the internal pool of materials made available from the above processes, provides a second opportunity for the selection between an amino acid and its analog for protein synthesis. Further competition among these materials occurs at the time, or prior to the time, of incorporation into the internal pool. Once an amino acid, or an acceptable analog, has been incorporated into this pool no further dilution or exchange with other cellular or extracellular amino acid carbon occurs^{14,15}.

The yeast cells (Table II), given an external ratio of analog to amino acid of 10/1, contained these materials in the expandable pool at a ratio of 5.2/1. The ratio of the analog to amino acid in the internal pool was found to be 2.3/1, and was identical to the ratio obtained in the proteins. *The final selection between natural amino acid and analog for protein incorporation thus must occur at the time of formation of the internal pool.*

Environmental conditions may markedly affect the degree to which analog substitution occurs, but the amounts of analog in the internal pool appear closely related to the final protein composition. One wonders whether the amino acids and analogs contained in the internal pool have not already been selected by the protein-forming templates, but have yet to be linked together in polypeptide strands.

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E. Summary of E. coli Pools

Our investigations of metabolic pools in E. coli fall into three categories. First there was a qualitative study of the properties of amino acid and nucleotide pools described in sections II.B.1 and II.C.1. Next came an intensive examination of many aspects of amino acid pools and their interpretation in terms of a carrier model described in section II.B.2. Finally the nucleotide pool was re-examined to evaluate its influence on the kinetics of the incorporation of tracers into nucleic acid (II.C.2 and II.C.3). This section will attempt to interpret the differences and the similarities exhibited by amino acids and nucleotides in terms of a common model.

Three aspects of the amino acid pools that determine the basic features of the carrier model are summarized in tabular form. Table 1 lists observations relating to maintenance or loss of amino acids from the pool. Clearly the pool amino acid does not leave the cell promptly after removal of the conditions required for its rapid concentration. It thus appears that the pool size reaches a steady value as a result of a decrease in the inward flux of amino acid. If the steady state were reached as a result of outward flux rising to balance the large initial inward flux, the conditions listed in Table 1 would cause a rapid loss from the pool.

TABLE 1
STABILITY OF THE PROLINE POOL IN *E. coli*

<i>Each of these conditions:</i>	<i>Leads to these results:</i>
Absence of energy source	{ Suppresses increase of pool Maintains it at pre-established level Permits exchange at a rapid rate
Low temperature (0-5 C)	
Reduction of proline concentration	
<i>However</i>	
Osmotic shock	{ lead to loss* of the pool
Freezing	
Mild reagents or	
Small pH changes	

*Without necessarily interfering with the growth of the cells or their rapid recovery of normal growth rate after return to normal culture conditions.

TABLE 2
EVIDENCE FOR A CATALYTIC STEP IN THE AMINO ACID CONCENTRATING PROCESS

1. Isoleucine interferes with valine uptake at a concentration far below that which gives a maximum isoleucine pool.
2. Rate of pool formation is maximal at an external concentration far below that at which the pool size becomes maximal.
3. Exchange rate at 0 C is almost independent of external concentration but is proportional to pool size.

Table 2 summarizes some evidence that there exist stereospecific sites which act as catalysts for the concentration of amino acids by the bacterial cell. Isoleucine and valine, when present at high external concentration, give rise to relatively large pools. When isoleucine is present at a moderate concentration yielding a small isoleucine pool, however, it strongly inhibits the uptake of valine present at low concentrations (Figure 7, section II.B.2). Thus the common step in the concentration process is very nearly completely saturated with isoleucine. This demonstrates the existence of something equivalent to a catalytic site and further that this site is saturated at an external concentration far below that giving a maximum pool.

TABLE 3
EVIDENCE FOR MORE THAN ONE COMPONENT IN THE POOL

-
- | |
|--|
| 1. Exchange at 0 C shows two components with widely different rates and sizes. |
| 2. Curve for pool size as a function of external concentration (formation at 25 C) shows two components. |
| 3. Small pools are specific while large pools are unspecific. |
-

Table 3 summarizes the evidence that there exists more than one component in the pool of a given amino acid. The observed time course of proline exchange at 0°C does not follow a simple exponential. When experiments covering a wide range of concentrations and pool sizes are examined, at least two components with widely different time constants can be resolved. Since the only process occurring is the trading of a labeled molecule in the pool for an unlabeled one in the environment, a simple exponential decay of pool radioactivity would result if there were a single component in the pool. Thus it may be concluded that there are at least two separate components. At low concentrations of proline the rate of uptake and the small pool size reached are not influenced by the presence of fifteen other amino acids, each at 100 times the proline concentration. However, the maximal proline pool reached at high concentrations of external proline is strikingly reduced in the presence of other amino acids. This is consistent with the presence of two components and even suggests that a different and less specific mechanism plays a role in the formation of large pools.

The two (or possibly more) components of the proline pool must be associated with the cell in different ways. If the pool were assumed to be in the form of unbound amino acid, the existence of a second impermeable membrane would have to be postulated. In the carrier model the two components of the pool are assumed to be associated with two groups of sites having different affinities for the amino acid.

The carrier model was developed to account for these striking features of the amino acid pools. The formation or expansion of the pool is governed by quite different factors from those determining the maintenance of an existing pool. The concentration of amino acids from the medium to form a pool requires metabolism. In contrast, a pool can be maintained at its preexisting level even when the energy source is removed or the temperature is reduced. Furthermore, exchange can continue at a rapid rate under these conditions.

These properties indicate that entry of an amino acid into the cell and its subsequent storage are separate and distinct. In the carrier model, amino acids are postulated to attach to a stereospecific carrier molecule and subsequently be transferred to storage sites. Thus saturation of the rate of entry indicates saturation of the carriers, and this can occur at concentrations which do not ultimately saturate the storage sites.

The carrier is postulated to play a more general role than simply to move amino acids from the medium to the storage sites. It picks up amino acids at their site of synthesis and transfers them to sites of protein synthesis, in this way regulating the rate of amino acid synthesis. Equations describing the carrier model are derived in full in section II.B.2, page 51, while its application to amino acid pools is described in section II.B.2, page 55, and its application to nucleotide pools in section II.C.2.

The differences and similarities between the observations of the behavior of amino acids and nucleic acid bases in *E. coli* may be summarized as follows: (1) The amino acids are not chemically converted to any great extent except in the usual pathways of amino acid synthesis, but the bases are promptly converted to nucleoside phosphates. (2) Amino acid pools in the absence of supplement may be very small while the nucleosidephosphate pools are large. (3) The amino acid pool size is responsive to the external concentration, but the nucleoside phosphate pools are not influenced by the base concentration. (4) Amino acids may be taken up by the cell at 10 times the rate required for protein synthesis, whereas bases are not taken up at a greater rate than that required for formation of RNA. (5) Radioactivity from exogenous amino acids is diluted by the pool before entering protein whereas that from exogenous bases may bypass the nucleoside phosphate pools and enter RNA without delay. (6) Exchange plays an important role in the labeling of amino acid pools but a negligible role in the labeling of the nucleoside phosphate pools. (7) Both amino acids and bases are taken up effectively at low concentrations. (8) Internal synthesis is effectively suppressed at moderate concentrations of the bases and certain of the amino acids. (9) Both are efficiently incorporated into macromolecules in the growing cell.

In spite of these striking differences, it is only necessary to alter a few of the reaction rate constants to make the predictions of the carrier model agree with the behavior of nucleic acid bases. When amino acids are supplied to the cell, the amino acid itself is supposed to form a complex with the carrier, whereas in the case of nucleic acid bases the base becomes a part of a molecule (presumably a nucleoside phosphate) attached to the carrier. This process is rapid, occupying less than a few seconds, and the resulting chemical form is apparently suitable both for incorporation into nucleic acid and for exchange with the nucleoside phosphate storage pools.

For amino acids the storage sites are taken to be only partially occupied, and pool expansion results when amino acid supplements are present. When nucleic acid bases are supplied, even though internal synthesis of the bases is shut down, no expansion of the pool occurs. Thus it must be assumed, in the carrier model, that the storage sites are almost entirely saturated with nucleoside phosphates in the absence of supplement.

For amino acids, when pool expansion occurs, the rate of uptake can be very much larger than the rate of utilization for protein synthesis. But for nucleotides, with all the storage sites initially occupied, the rate of uptake is limited to the rate of utilization.

The rate of exchange between carrier-associated amino acid and that associated with storage sites is taken to be so fast that under most experimental conditions isotopic equilibrium occurs. Radioactivity therefore enters protein at a rate determined by the specific radioactivity of the pool. On the other hand, for nucleotide pools the rate of exchange must be taken to be relatively slow between carrier-associated and storage site-associated compounds. The fact is that labeled bases may be transferred from the environment or from their site of internal synthesis to the sites for utilization for nucleic acid synthesis without completely mixing with the large storage pools. The carrier, proposed for amino acids where no such separation was evident, exactly fills the required role of a partially isolated transfer system.

All the observations relating to nucleotide pools can be explained using the carrier model without the addition of any new elements. This is a pleasing

result and suggests that there is a kernel of truth (if only symbolic) in the carrier model. When the next stage of understanding of pool mechanisms is reached, the carrier and the sites may have disappeared but the equations derived from these concepts will remain correct as a first approximation.

Roy J. Britten.

F. Summary of Yeast Pools

Our studies of the metabolic pools of yeast were initiated to search for peptides which at that time were considered possible precursors of protein. Candida utilis was chosen because it was known to contain large quantities of low-molecular-weight materials that could be used for protein synthesis. No significant quantities of peptides were found, and there was no indication that they played any part in protein synthesis. The experience gained, however, did indicate that one large amino acid pool was a compulsory intermediate in protein synthesis. Concurrent studies of pools in E. coli showed important differences from the pools of yeast. As E. coli and yeast differ greatly in the complexity of their internal structures (E. Vitols, R. J. North, and A. W. Linnaue, J. Biophys. Biochem. Cytol., 9, 689-699, 1961), comparative studies might indicate features of pools that could be attributed to structure. The differences between these organisms became more striking as the studies developed. Furthermore, some of the features observed in yeast are difficult to interpret without invoking effects of structure.

Heterogeneity is one of the most striking features of the metabolic pool in yeast. In E. coli different components can be discerned, but in yeast they are unmistakable. The first example of heterogeneity was found in studies of purine utilization (II.C.2). Cells growing with sugar as the sole source of carbon contain roughly 20 μ moles per gram dry weight of nucleotides but no free bases. When adenine is added to the medium, it is concentrated to form a free base pool. This adenine must be converted to the nucleotide form before it can be further changed to guanine or incorporated into nucleic acid. However, the formation of the adenine pool does not affect the size of the purine nucleotide pool.

Thus it appears that chemically distinct and functionally different pools are present. The first, a concentrating pool, accumulates nucleic acid bases within the cell to levels exceeding the external concentrations. This pool is evident only when the synthetic medium is supplemented with bases. Its size is variable and dependent upon the external concentration and form of the exogenous supplements. Once concentrated, these bases may provide material for the second, a nucleotide pool, which is always present, remains constant in size, and, in the absence of exogenous supplements, is derived solely from fructose (or other sugars). Here conversion of one nucleotide to another occurs, furnishing the appropriate molecules for nucleic acid synthesis.

The pool of bases has been designated the expandable pool or preferably the concentrating pool, and the nucleotide pool has been designated the internal or conversion pool. Similarly studies of the amino acid pools show analogous characteristics even though no clear distinction between the chemical forms of the molecules held in the different pools could be detected.

The concentrating pool provides an intracellular accumulation of amino acids to levels exceeding their external concentrations. The size of this pool varies with external concentration and is seen only in the presence of exogenous amino acids. The components of this pool exchange with external amino acids, are extractable with cold water, and are sensitive to osmotic shock.

The second pool, called conversion pool, is present in growing cells in which the amino acids are synthesized from sugar and inorganic nitrogen. The conversion of one amino acid to another occurs in this pool before they are withdrawn for protein synthesis. Neither exogenous amino acids nor components of the concentrating pool affect the size of the conversion pool or the transfer of these amino acids to proteins. During exponential growth this pool remains constant in size and contains 13 per cent of the total cellular carbon and 10 per cent of the total cellular nitrogen (H. Halvorson, W. Fry, and D. Schwemmin, *J. Gen. Physiol.*, **38**, 549, 1955). The conversion pool components are insensitive to osmotic shock and, together with the concentrating pool components, are extractable with cold trichloroacetic acid (TCA). Although exogenous amino acids do not compete with the flow of preformed conversion pool components to protein, they do repress the formation of conversion pool components from sugars. This inhibition must therefore occur before the amino acids enter the pool.

The properties of the concentrating pool are described in II.C and have been summarized by Halvorson and Cowie (Symposium on Membrane Transport and Metabolism, published by Czechoslovak Academy of Science, 1961). In general this pool is similar to the amino acid pool of *E. coli*. Many of its features (including a bypass) can be interpreted in terms of a carrier model (II.D).

In *E. coli* however, amino acids pass from the pool directly to the sites of protein synthesis. In *C. utilis* they must enter the conversion pool as a compulsory stage. Thus the conversion pool which appears to be lacking in *E. coli* (or possibly is too small to observe) is more likely to be an expression of the increased structural complexity of yeast.

Earlier studies of the biosynthesis of amino acids (Studies of Biosynthesis in *Escherichia coli*, CIW Publication 607) demonstrated the grouping of amino acids into families. Thus in *C. utilis* glutamic acid is the precursor to arginine, proline, and lysine; aspartic acid is the precursor to threonine, methionine, and isoleucine.

When *C. utilis* is exposed briefly to C^{14} fructose, a large fraction of the radioactivity appears in the amino acids of the conversion pool. Furthermore, the radioactivity is initially restricted to the amino acids of the "family heads." As growth continues, using C^{12} fructose, the radioactivity of the family head amino acids is transferred partly to protein and partly to the product amino acids of the pool. No amount of exogenous C^{12} amino acids affects these transfers of radioactivity even though the amino acids rapidly accumulate to form a large concentrating pool.

If, however, the C^{12} amino acids are supplied concurrently with the C^{14} fructose they can reduce the quantity of radioactivity found in the conversion pool and in the protein. Evidently, exogenous amino acids can compete with endogenously formed ones for entry into the conversion pool but not with the amino acids already present in the pool.

C^{14} glutamic acid, already in the conversion pool, is converted to C^{14} arginine and incorporated into protein with equal rapidity whether or not C^{12} arginine is added to the medium. In contrast, C^{12} arginine can prevent the formation of C^{14} arginine from C^{14} fructose if the C^{12} arginine is added at the same time as the C^{14} fructose.

One interpretation of these complicated interactions is that either glutamic acid or arginine can enter the arginine system, but once the glutamic acid has entered, it cannot be displaced. Thus roughly one-fourth of the glutamic acid of the conversion pool seems to be committed to arginine formation.

Protein synthesis utilizes exclusively the amino acids from the conversion pool. Other amino acids held within the cell do not mix with those en route from the conversion pool to the sites of protein synthesis. Discrimination between phenylalanine and parafluorophenylalanine can occur on entry into the concen-

trating pool, and a further selection happens on entry into the conversion pool. However, the ratio of one to the other is the same in the protein as in the conversion pool, indicating no further selection at the site of protein synthesis (II. C.4). It, therefore, appears that amino acids emerge from the conversion pool in an activated form ready for incorporation into protein, and it is this difference in their chemical state that prevents mixing with other amino acids.

Observations of the flows of amino acids through the pools to the protein are summarized in Fig. 1. Such a diagram provides a group of facts to be explained but no indication of the mechanisms involved. The concept that the amino acids are simply held within the cell by an impermeable cell wall or membrane is clearly untenable, since at least two pools having markedly different characteristics coexist in the same space.

The concept of carriers and adsorption on sites is useful but perhaps not sufficient. Partially specific carriers may be involved in entry. The same car-

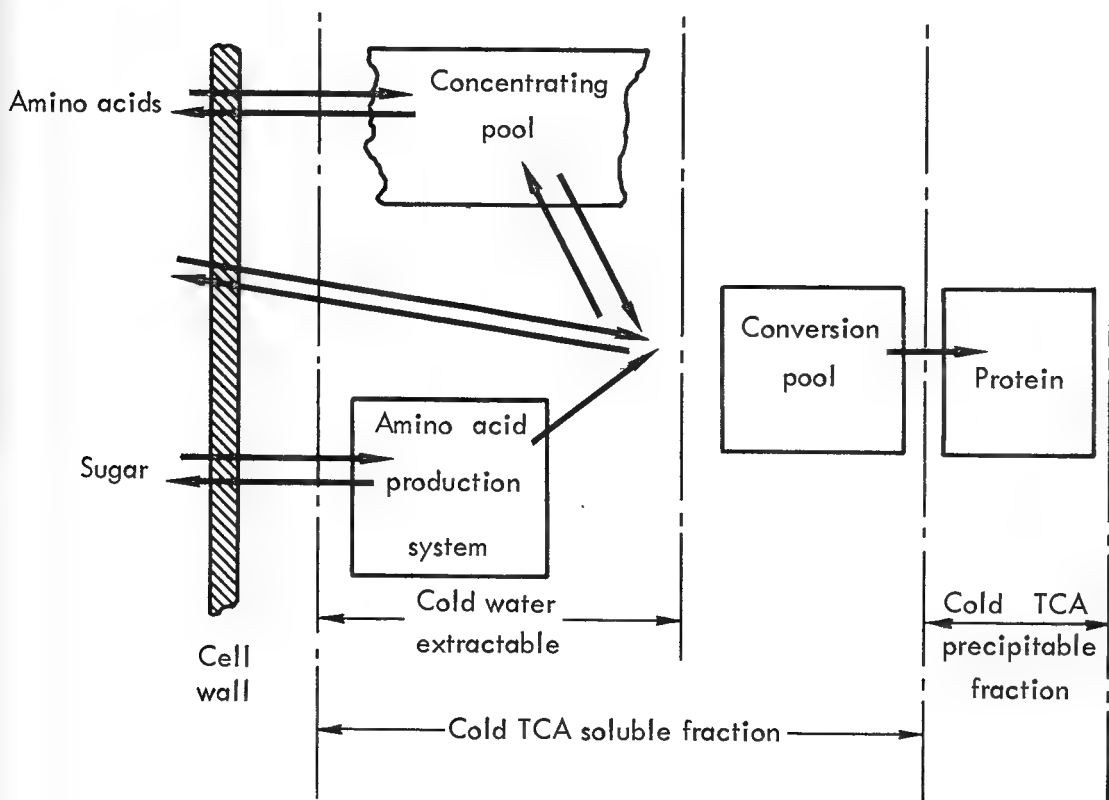


Fig. 1. Carbon flow in yeast.

riers or simple diffusion of free amino acids can account for movement from sites of synthesis to pools, and between the different pools. A different carrier to which the amino acids are complexed in high energy states (as exemplified by the S-RNA-amino acid complex) seems inescapable for the transit from the conversion pool to the site of protein synthesis.

Only a small fraction of the amino acids could be held by carriers at any one time. There simply are not enough molecules of low molecular weight available in the cell. If the complex has a high turnover, however, the function could be carried out by the small quantities available.

Adsorption on sites is also a useful concept, as it might provide specificity and hold amino acids in the definite quantities found in the conversion pool. Again, the large quantities of amino acids rule out the classes of molecules likely to act as adsorbers. The amino acids of the conversion pool exceed the total nucleotides of nucleic acid. Neither does it seem likely that the proteins alone would adsorb one-third of their weight as amino acids.

These difficulties in finding sites capable of holding amino acids by adsorption suggest that the structure of the cell must be involved. An extension of the model should retain the features of specificity and finite size and provide additional holding capacity. Small regions in which the amino acid conversion systems were organized and which held high concentrations of their substrates and products would account for many of the features of the conversion pool. Small carriers could transport each amino acid to its appropriate region. Entry could be specific and limited because of the properties of the receptors. The amino acids would emerge only after they had undergone conversion and activation and were ready for diffusion to the polysome sites of protein synthesis. Other regions of the cell lacking the conversion and activating enzymes and having less structural stability could hold the materials of the concentrating pools.

Methods of breaking cells and fractionating their components have been improved so that it is now possible to observe structures as fragile as polysomes. Perhaps the isolation of intact vesicles still containing their store of pool materials will some day be achieved. Dean B. Cowie.

III. RIBOSOMES

A. General Properties of Ribosomes

III.A.1 Excerpt from 'Kinetic Studies of the Synthesis of RNA and Ribosomes'

(Reprinted, by permission, from Molecular Genetics, pp. 292-311, 350-352, Academic Press, New York, 1963.)

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I. INTRODUCTION

A. MICROSOMES AND RIBOSOMES

The term "microsome" was originated by Claude in 1943 to describe a fraction obtained by differential centrifugation of disrupted vertebrate cells (Claude, 1943). After breakage, the cells were centrifuged at low g to sediment nuclei, cell walls, and mitochondria. The supernatant fluid, devoid of structures visible in the microscope, was then centrifuged at 100,000 g for 2 hours and the pellet so obtained was designated the microsomal fraction. In it appeared much of the cytoplasmic ribonucleic acid (RNA) and lipid.

Electron microscopy of this pellet showed that it consisted mainly of membranes to which were attached dense particles of about 200 Å diameter. Electron micrographs of the same cells showed an extensive network of membranes designated the endoplasmic reticulum. In addition, dense particles could be seen sometimes free and sometimes attached to the membranes. The RNA content of the pellet was attributed to its content of particles (see review, Palade, 1958). Particles from bacteria had been observed earlier (Luria *et al.*, 1943.)

Analysis of the microsome fraction in the analytical centrifuge, especially after treatment with deoxycholate to dissolve the membranous material, showed the presence of objects having sedimentation coefficients in the range 20–100S (Peterman and Hamilton, 1957).

Similar fractions of high RNA content were obtained from bacteria, yeast, and plant cells. All showed the presence of a number of discrete peaks in the analytical centrifuge having sedimentation coefficients of 20–100S (Schachman *et al.*, 1952; Chao and Schachman, 1956; Ts'O *et al.*, 1956).

Since the microsomal fraction included both the membrane material

and the RNA-rich particles as well as any protein which was sedimented by the prolonged centrifugation, the reported composition of the microsomes was highly variable. In 1957 the term "ribosome" (originally suggested by H. M. Dintzis) was introduced to distinguish the particulate material from the remainder of the microsome fraction (Roberts, 1958).

B. EFFECTS OF MAGNESIUM

In the same year the effect of magnesium concentration upon the sedimentation coefficients obtained for the ribosomes was recognized. Chao showed that the larger ribosomes (about 80S) obtained from yeast

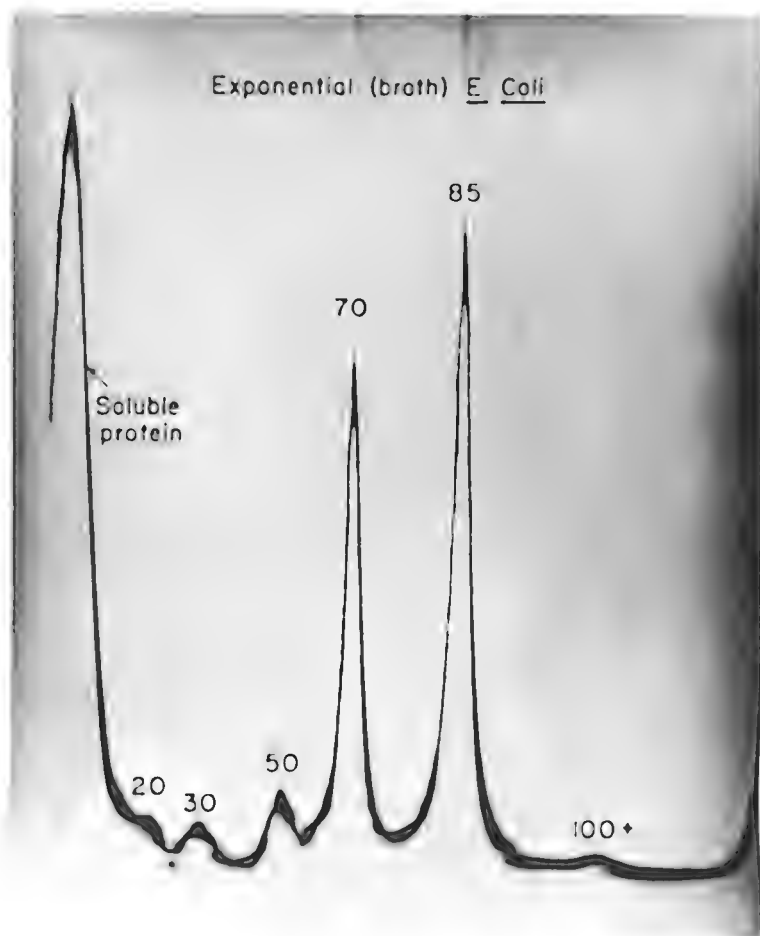


FIG. 1. Centrifugation pattern of juice from *E. coli* growing in broth medium. These rapidly growing cells have a high content of ribosomes. Sedimentation from left to right observed in Spinco Model E ultracentrifuge.

dissociated into two components of about 60S and 40S when the magnesium concentration of the suspending fluid was reduced from $10^{-8} M$ (Chao, 1957). Bolton *et al.* independently found a similar effect in the ribosomes of *Escherichia coli* (Bolton *et al.*, 1958). Ts'O *et al.* reported the dissociation of the 80S component of ribosomes of pea seedlings into

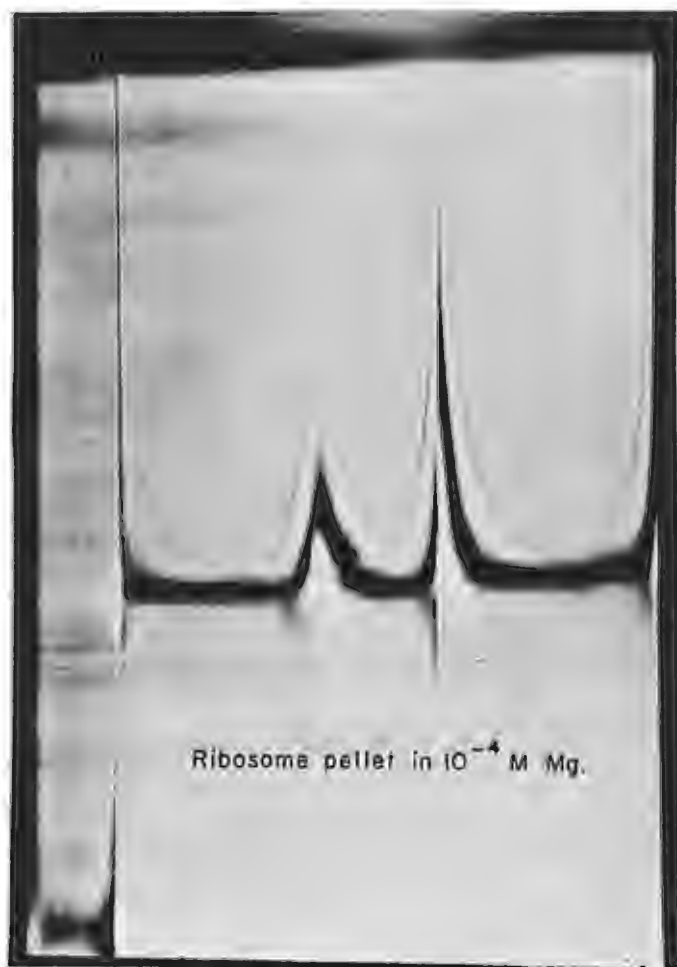


FIG. 2. Centrifugation pattern of ribosome pellet in $10^{-4} M$ Mg. Only 50S and 30S ribosomes appear.

components of 60S, 40S, and 26S (Ts'O *et al.*, 1958). A Mg/P ratio of 0.3 was required to preserve the integrity of 80S particles from pea seedlings (Edelman *et al.*, 1960).

The sedimentation coefficients reported in early papers vary widely; most of the values were not corrected for viscosity or concentration.

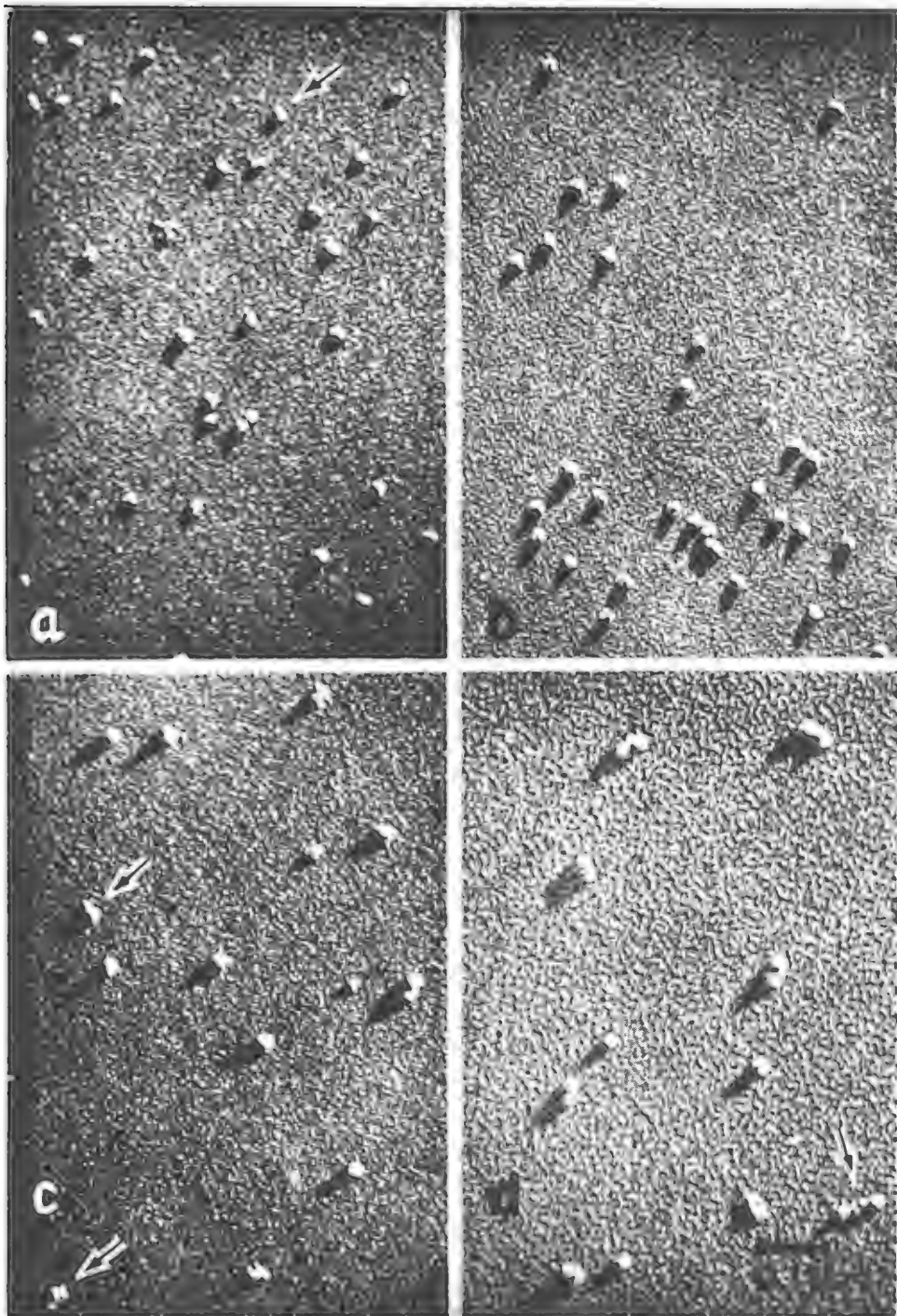


FIG. 3. Ribosomes of *E. coli* ($\times 100,000$). (Hall and Slayter, 1959.) (a) 30S preparation, (b) 50S preparation, (c) 70S preparation, (d) 100S preparation.

When these corrections are made the ribosomes of *E. coli* show three prominent groups at $30.6 \pm 1S$, $50.0 \pm 1S$, and $69.1 \pm 1S$. These are usually rounded off to 30, 50, and 70S for identification (Tissières *et al.*, 1959).

Ribosomes extracted from *E. coli* at $10^{-2} M$ Mg show predominantly the 70S (or larger) form (Fig. 1), but when the extraction is carried out in $10^{-4} M$ Mg or when the Mg concentration is reduced to $10^{-4} M$ after extraction the large particles give rise to equal numbers of 30S and 50S (Fig. 2). This fact, together with electron microscope pictures which show shapes consistent with a 30–50S complex (Fig. 3), (Hall and Slayter, 1959; Huxley and Zubay, 1960) indicates that the 30S and 50S ribosomes associate reversibly to form 70S particles.

In addition, ribosomes of sedimentation coefficients greater than 70S are observed. One of these (about 100S) appears to be a dimer of the 70S particle (Fig. 3). The reactions are not simple, however. An intermediate group (called 85S for identification) also appears and its sedimentation coefficient as well as its proportions depends on the magnesium concentration (Britten and McCarthy, 1959). As this 85S group yields 30S and 50S particles in equal numbers when the magnesium concentration is reduced, it appears to be due either to an altered shape or hydration of the 70S or 100S particles or to a rapid equilibration between the 70S and 100S forms which yields an intermediate sedimentation coefficient.

C. OTHER INFLUENCES ON RIBOSOME PATTERNS

The sedimentation pattern also depends upon the metabolic state of the cell at the time of breaking and upon the method of breaking the cells (Fig. 4). McCarthy has shown that cells rapidly accumulate the 100S particles when their energy supply (glucose) is exhausted and revert with equal rapidity to the normal pattern when glucose is restored (McCarthy; 1960). Similar changes also appear when mutants are deprived of a required metabolite. There is little difference, however, in ribosomes taken from different phases of the growth cycle in synchronized cultures (Britten *et al.*, 1960).

Prolonged incubation of growing cells in Mg-free media reduces the ribosome content to less than 5%. The ribosome content is restored (following an exponential growth curve) when the magnesium of the medium is replenished (McCarthy, 1962).

The ribosome pattern does not result simply from an equilibrium among two basic units and combination thereof. Some 30S and 50S particles are present in the cell juice even when the magnesium concentration is high. These are sometimes designated "native" 30S and 50S

ribosomes in distinction to the "derived" 30S and 50S particles which are obtained from the dissociation of 70S particles. Native particles have higher specific radioactivities (after a short period of exposure to the tracer) than do the derived particles (Britten and McCarthy, 1959). Isolated native particles do not combine (Green and Hall, 1961).

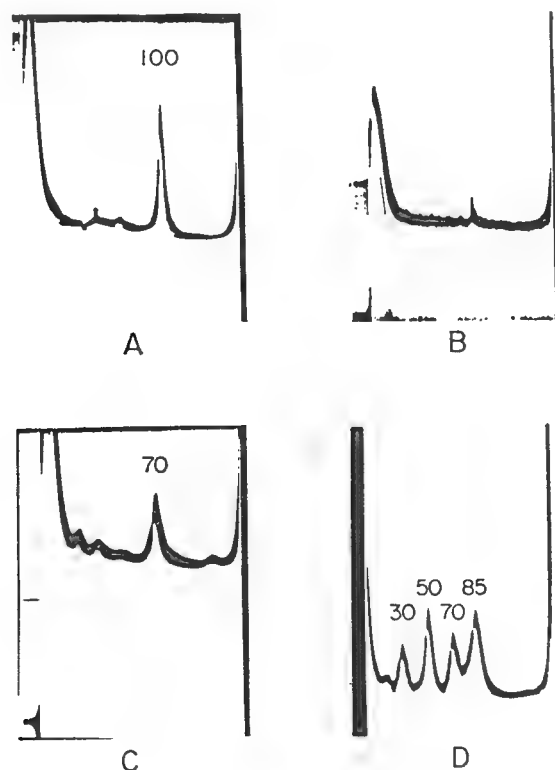


FIG. 4. Changes in ribosome pattern caused by different metabolic conditions: A. The 100S component is prominent when growth is stopped by lack of energy source. B. Magnesium starvation causes disappearance of ribosomes. C. Incubation with chloramphenicol results in accumulation of small particles. Ribosomes already present appear as 70S. D. Incubation with 5-fluorouracil results in accumulation of 30S and 50S ribosomes.

Furthermore, it has been shown *in vivo* (McQuillen *et al.*, 1959) and *in vitro* (Tissières *et al.*, 1960) that the 70S particles carrying newly made protein do not dissociate completely when the magnesium concentration is reduced to $10^{-4} M$.

In addition to the main peaks which are readily observed smaller groups of particles sometimes appear. Many preparations show a peak at about 20S. The 20S component is complex, containing one component

rich in protein and another rich in RNA which may be a ribosome precursor. The 20S particle also appeared after prolonged storage of ribosomes at 10^{-4} M magnesium. In this circumstance a sufficient quantity was obtained to show that the protein/nucleic acid ratio was about 40/60 but it is not known whether this degradation product is the same as the particle observed in cell juices (Roberts and Duerksen, 1960; Aronson and McCarthy, 1961).

Another particle about 43S plays a prominent role as a protein deficient precursor to the 50S ribosome (see Section V). It has also been observed in the juice of cells in which DNA synthesis was prevented by lack of thymine (Roberts, 1960) and as a degradation product of 50S ribosomes (Elson, 1961).

Abnormal patterns develop when cells are exposed to antibiotics or analogs. Chloramphenicol causes the accumulation of small particles deficient in protein while preventing the entry of P^{32} into the normal particles (Pardee *et al.*, 1957; Nomura and Watson, 1959; Bolton, 1959). 5-Fluorouracil causes the accumulation of 30S and 50S ribosomes which are unable to form 70S particles (Aronson, 1961).

A part of the complexity of the ribosome pattern may be due to the association of newly synthesized RNA or protein with the particles (see Section V,C and VIII). Such material might facilitate or inhibit association of the basic units. In most experiments its presence would be undetected and uncontrolled, thus giving rise to unpredictable variations.

D. GENERALITY OF RIBOSOME PATTERNS

Because of the wide range of ribosome patterns which can be observed in one organism it is difficult to compare the ribosomes from different sources. Those which have been most extensively studied show in common particle groups of 70–100S which dissociate in low concentrations of magnesium into two groups of 26–40S and 40–60S. McCarthy has surveyed the ribosomes derived from a number of microorganisms and found a general similarity of patterns (McCarthy, 1959). Some minor differences appeared but they cannot be considered significant without extensive studies of the effects of growth conditions. Also, the magnesium concentration required for stability varies from one organism to another.

In view of these difficulties of intercomparing ribosomes and because most of the studies of ribosome synthesis have been carried out with *E. coli*, it seems best to describe the process of ribosome synthesis as it has been observed in this organism. We hope that this will prove to be a specific example of a general process. Furthermore, we have omitted any reference to the synthesis of RNA in virus-infected cells as the relation of this process to ribosome synthesis is still obscure.

E. RIBOSOMES AS SITE OF PROTEIN SYNTHESIS

Interest in the biosynthesis of ribosomes stems primarily from the belief that the ribosomes serve as the principal machinery of the cell for protein synthesis. The evidence favoring this view has accumulated over a period of years and is now quite convincing. Cassperson and Brachet observed that RNA was invariably present in high quantity wherever or whenever there was a high rate of synthesis (Cassperson, 1950; Brachet, 1950). Caldwell and Hinshelwood showed a quantitative correlation between the quantity of RNA and the growth rate of a wide range of different cells and different conditions (Caldwell and Hinshelwood, 1950). Subsequently, when it was found that most of the RNA was located in ribosomes, the same evidence indicated that a high concentration of ribosomes was correlated with a high rate of protein synthesis.

Further evidence appeared in the studies of incorporation of labeled amino acids into protein. *In vivo* experiments showed that the radioactivity of the microsome fraction has a rapid initial rise, whereas that of the soluble proteins has an initial delay suggesting a precursor product relationship (Borsook *et al.*, 1950; Littlefield *et al.*, 1955). In mammalian tissues the times involved were about 15 minutes and in bacteria the times were about 15 seconds. Furthermore, with the bacteria it was possible to follow the "pulse" of radioactivity with a "chase" of non-radioactive material and to show a transfer of radioactivity from the ribosomes to the soluble protein (McQuillen *et al.*, 1959).

In vitro experiments showed that ribosomes were an essential component of any cell-free system capable of incorporating amino acids into protein (Littlefield and Keller, 1957; Tissières *et al.*, 1960). In these cell-free preparations a large part of the radioactivity incorporated into peptide linkage remained associated with the ribosomes.

It is now widely accepted that ribosomes furnish the sites of protein synthesis and that the information for the assembly of the amino acids in their proper order is transferred either to the RNA of the ribosomes or to an RNA which is associated with the ribosomes while it acts as template for protein synthesis.

II. PROPERTIES OF RIBOSOMES *in Vitro*

A. PREPARATION AND PURIFICATION OF RIBOSOMES

The preparation of ribosomes begins with the harvesting of the cells. A culture in the desired metabolic condition is rapidly chilled to maintain that state and centrifuged. The resulting pellet is washed three times to replace the growth medium by a buffer suitable for subsequent

processing of ribosomes. The buffer most frequently used is tris(hydroxymethyl)aminomethane 0.01 *M* pH 7.4 with magnesium added as the chloride at the desired concentration (usually 10^{-2} *M*).

The cells may then be broken by grinding the pellet with twice its weight of alumina, by repeated freezing and thawing with lysozyme, or by forcing them through a small orifice in the French pressure cell. The pressure cell is preferable for the preparation of ribosomes as it is highly efficient in breaking most cells and the DNA is degraded so that the cell juice is not viscous.

The cell juice is then diluted to less than 100 mg wet weight/ml and the cell walls, membranes, and any unbroken cells are removed by centrifugation for 1 minute at 40,000 rpm in the angle head rotor of the Spinco Model L ultracentrifuge. The supernatant fluid (40K 1'S) is decanted and the pellet is either discarded or resuspended to recover its content of ribosomes (about 10–20%).

The crude ribosome pellet is then obtained by centrifugation of the 40K 1'S. If complete recovery of ribosomes as small as 30S is required, 2–3 hours of centrifugation is needed. Alternatively, if only a 90% recovery of 70–100S ribosomes is desired, 30 minutes' centrifugation will suffice.

The first ribosome pellet is highly contaminated, partly by small bits of cell wall and membrane and partly by soluble protein and RNA. Much of the contaminating material will not resuspend readily and can be removed by a brief centrifugation (40K 1'). Alternate short and long cycles of centrifugation helps to reduce the contamination and the ribosome pellet becomes more colorless and transparent. By appropriate choice of the times of centrifugation, pellets rich in one or another class of ribosome can be obtained. However, this technique is not adequate for measurements of the protein or enzyme content of ribosomes. Further purification of the pellet by sedimentation through sucrose gradients or by column chromatography shows that after three cycles of sedimentation in the angle head rotor as much as half the protein content of the pellet may be due to contamination. The best use of differential centrifugation is to prepare partially purified ribosomes as the starting material for other techniques of separation.

B. PROTEIN/NUCLEIC ACID RATIO

Various values reported for the protein and nucleic acid content of ribosomes are listed in Table I. As many of these experiments were carried out before the difficulties of removing protein contaminants were fully appreciated, the protein content is likely to be overestimated. There is no certainty that the protein/nucleic acid ratio varies in

TABLE I
RNA CONTENT OF RIBOSOMES

Source of ribosomes	RNA (%)	Reference
Calf liver	40	Peterman and Hamilton (1957)
Yeast	42	Chao (1957)
Pea seedlings	40	Ts'O <i>et al.</i> (1956)
Rabbit reticulocytes	50	Dintzis <i>et al.</i> (1958)
<i>Escherichia coli</i>	60	Roberts <i>et al.</i> (1958)
<i>Escherichia coli</i>	63	Tissières <i>et al.</i> (1959)

different species; the reported variations may be due to variable contamination.

Ribosomes of different sedimentation constants appear to have the same protein/nucleic acid ratio. Figure 5 shows the correspondence between the protein (measured by S^{35}) and the nucleic acid (measured by

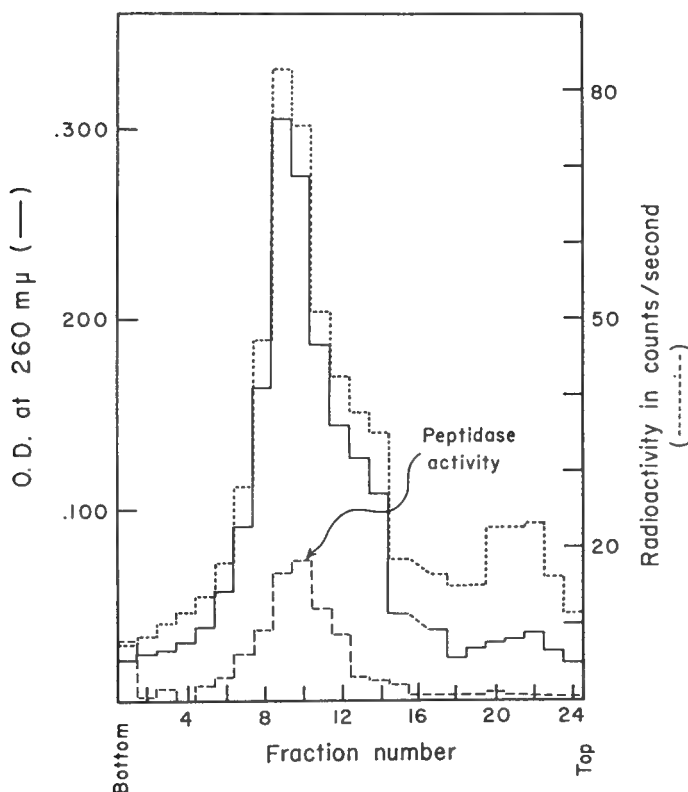


FIG. 5. Sedimentation analysis of a ribosome pellet shows correspondence between nucleic acid content indicated by optical density (O.D.), protein indicated by S^{35} , and peptidase activity.

optical density) in ribosomes separated by sedimentation. Similar results are shown in Fig. 6 using P^{32} and C^{14} -leucine. Thus the ribosomes of *E. coli*, whether 30S, 50S, 70S, or 100S, all have the same nucleic acid

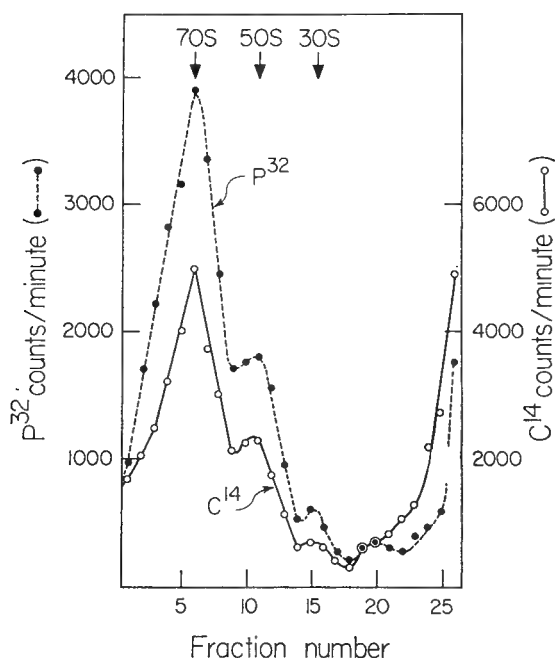


FIG. 6. Sedimentation analysis of a total cell extract from *E. coli* cells in tris-HCl 0.01 *M*, pH 7.4, $MgCl_2$ 0.01 *M*, randomly labeled with C^{14} -leucine and $P^{32}O_4^{--}$. Centrifugation 90 minutes at 37,000 rpm, 4°C. Note same ratio of protein/nucleic acid in ribosomes of different sedimentation coefficients. Protein indicated by radioactivity of C^{14} -leucine, nucleic acid indicated by P^{32} .

content of 63% or possibly higher. Notable exceptions to this statement are the particles which accumulate during growth in chloramphenicol and the precursors of the ribosomes (see Sections V–VII).

C. PROTEIN COMPONENTS

1. Structural Elements

The protein and nucleic acid components of ribosomes are readily dissociated either by treatment with 4 *M* urea or by removal of magnesium with chelating agents such as disodium ethylenediaminetetraacetate. Bolton showed the presence of several components separable by chromatography (Bolton, 1958). More extensive studies by Waller and Harris show at least 20 components which are resolved by electrophoresis.

Methionine (about 47%), alanine (about 37%), and serine (about 11%) account for most of the NH_2 terminal amino acids and the average molecular weight of the individual protein components estimated from the proportion of NH_2 terminal amino acids is 25,000 (Waller and Harris, 1961). Since the molecular weight of the total protein of the 30S particle is only 260,000, any one 30S particle could include only about 10 of the observed components.

2. Ribosomal Enzymes

Latent ribonuclease (RNase) was found in chemically isolated ribonucleoprotein of *E. coli*. The enzyme was not active until the structure of the nucleoprotein was disrupted (Elson, 1958, 1959).

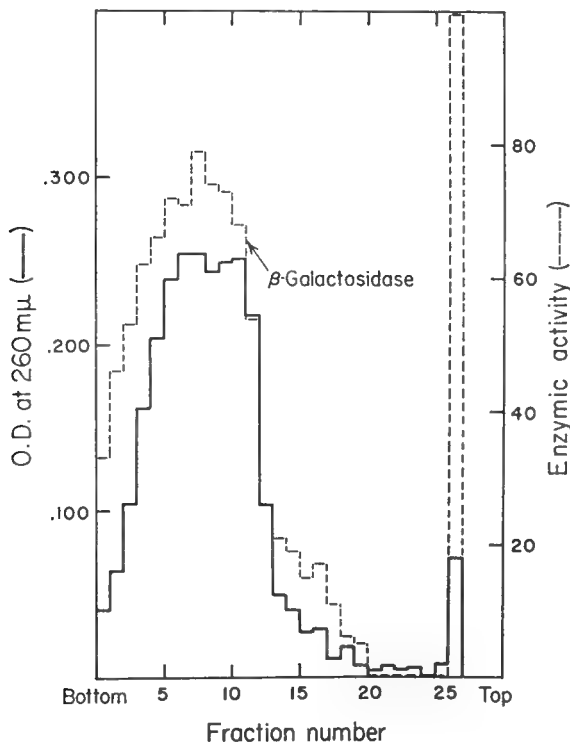


FIG. 7. Sedimentation analysis shows a correspondence between ribosomes indicated by optical density (O.D.) and β -galactosidase activity. (Cowie *et al.*, 1961.)

Latent RNase also appeared in ribosomes isolated by centrifugation or chromatography. Furthermore, the total cellular content of RNase could be attributed to the RNase of the ribosomes (Bolton, 1958). The enzyme is either localized in 30S particles exclusively (Elson and Tal,

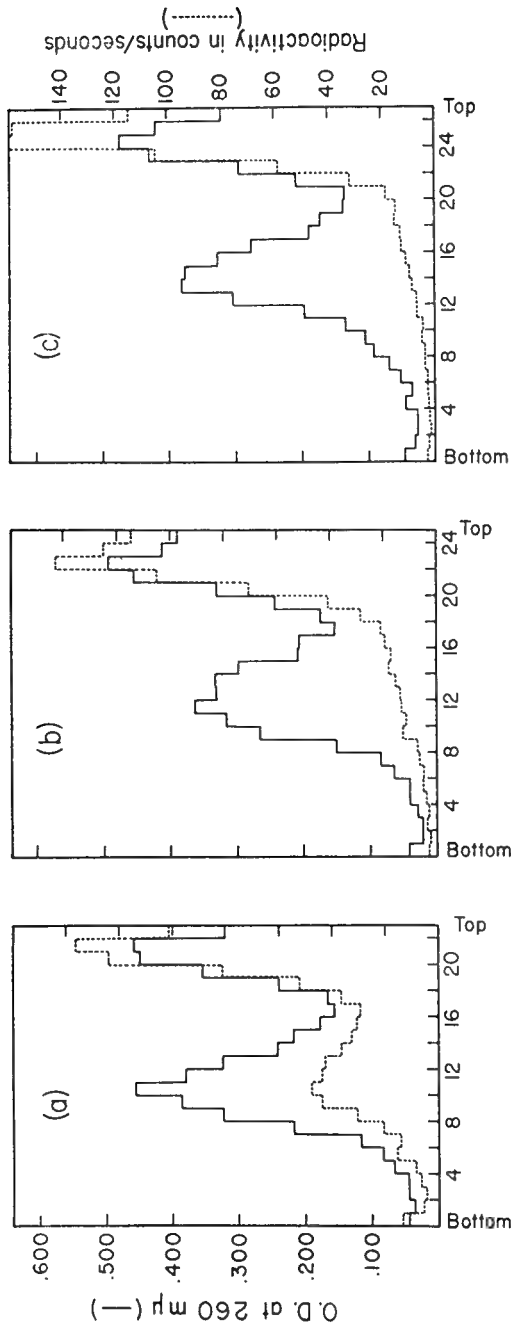


Fig. 8. Transient association of newly incorporated S^{35} with ribosomes shown by sedimentation analysis of total cell juice. (a) Cells incubated 15 seconds with $S^{35}O_4^{--}$, (b) 15 seconds' incubation with $S^{35}O_4^{--}$ followed by 15 seconds' incubation with S^{32} chaser, (c) 15 seconds $S^{35}O_4^{--}$ followed by 120 seconds with chaser. Note transfer of radioactivity from 70-85S region to nonsedimenting region.

1959; Spahr and Hollingworth, 1961) or equally distributed among the 30S and 50S particles (Bolton, 1959).

Deoxyribonuclease (DNase) is also present in ribosomes in a latent form (Elson, 1959).

The significance of other enzymes found in ribosome pellets is uncertain. The pellet is apt to be contaminated by soluble protein and adsorption of enzyme by ribosomes is difficult to rule out.

A major proportion of leucine amino peptidase was found associated with ribosomes of both 30S and 50S. The profile of enzyme activity corresponds to that of ribosomes in sedimentation analysis, ruling out simple contamination of pellets (Fig. 5). This enzyme shows no latency (Bolton and McCarthy, 1959).

Small traces of β -galactosidase continue to sediment with ribosomes after prolonged washing procedures (Fig. 7). Here again the activity of the ribosome associated enzyme is partially latent. Acid phosphatase and alkaline phosphatase have also been found in *E. coli* ribosomes (Cowie *et al.*, 1961).

3. Nascent Protein

Kinetic studies show the rapid appearance of newly incorporated S^{35} - and C^{14} -amino acids in ribosomes (Fig. 8). The quantity corresponds to about 0.1% of the total soluble protein, i.e., to the protein synthesized in a period of about 5 seconds (McQuillen *et al.*, 1959). In studies of the synthesis of the structural protein of ribosomes care must be exercised to distinguish the nascent protein, which is only transiently associated with ribosomes and not destined to become a part of their structure.

D. NUCLEIC ACID COMPONENTS

Degradation of the 50S ribosomes by sodium dodecyl sulfate or by phenol releases RNA of 16 and 23S corresponding to molecular weights of 0.55×10^6 and 1.1×10^6 . The 30S ribosomes release a single component of 16S (Kurland, 1960). Aronson and McCarthy report similar results and describe further a progressive degradation to small units of 13.1S, 8.8S, and 4.4S which was brought about by heating or removal of magnesium by dialysis. The 4.4S component was also found in the RNA extracted from magnesium starved cells (Aronson and McCarthy, 1961; McCarthy and Aronson, 1961). Further evidence for small subunits of ribosomal RNA shows in X-ray analysis (Timasheff *et al.*, 1961).

The nucleotide composition of ribosomal RNA has been reported by several authors and is given in Table II. Bacteria of different DNA composition show a total ribosomal RNA composition which is quite

TABLE II
NUCLEOTIDE COMPOSITION OF VARIOUS RNA's

Organism	DNA	S-RNA	70S	50S	30S	Reference
<i>Escherichia coli</i>	A 24	A	—	25.6	24.6	Spahr and Tissi�res (1959)
	T 24	U	—	22.1	21.0	
	G 26	G	—	31.4	31.6	
	C 26	C	—	20.9	22.8	
<i>Escherichia coli</i>	A 24	A 19.7	—	26.4	24.3	Bolton (1959)
	T 24	U 17.2	—	18.3	20.5	
	G 26	G 34.2	—	34.8	31.6	
	C 26	C 29.1	—	20.5	23.6	
<i>Escherichia coli</i>	A 24	A 20.5	25.1	25.4	24.8	Midgley (1962)
	T 24	U 16.5	20.4	19.6	21.5	
	G 26	G 33.2	32.6	33.5	31.0	
	C 26	C 29.8	21.9	21.5	22.7	
<i>Bacillus subtilis</i>	A 29	A 20.2	25.9	26.2	26.5	Midgley (1962)
	T 29	U 17.6	20.8	19.3	21.6	
	G 21	G 32.9	31.0	32.0	29.6	
	C 21	C 28.3	22.3	22.5	22.3	
<i>Proteus vulgaris</i>	A 32	A 19.1	26.2	26.5	24.7	Midgley (1962)
	T 32	U 18.3	20.7	20.8	20.4	
	G 18	G 33.3	31.4	31.4	31.9	
	C 18	C 29.3	21.7	21.3	23.0	
<i>Aerobacter aerogenes</i>	A 22	A 19.7	25.5	25.6	25.3	Midgley (1962)
	T 22	U 18.8	21.1	21.2	21.5	
	G 28	G 32.3	31.5	31.2	30.8	
	C 28	C 29.2	21.9	22.0	22.4	
<i>Pseudomonas aeruginosa</i>	A 18	A 20.8	25.7	26.3	25.1	Midgley (1962)
	T 18	U 17.1	21.0	21.3	20.5	
	G 32	G 33.8	31.6	31.2	32.8	
	C 32	C 28.3	21.7	21.2	21.6	

constant. The increased accuracy obtained by use of the isotope dilution technique shows a slight difference in composition between the RNA's derived from 30S and 50S ribosomes.

E. STRUCTURE OF RIBOSOMES

Table III lists the values of the physical constant of purified *E. coli* ribosomes as measured by Tissi res *et al.* Molecular weights of 0.9×10^6 and 1.8×10^6 for the 30S and 50S particles are consistent with the molecular weights of 0.55×10^6 and 1.1×10^6 found for the RNA, assuming an RNA content of 63%.

TABLE III
PROPERTIES OF *E. coli* RIBOSOMES^a

Particle	S	D	\bar{V}	η	MW ^b	MW ^c
30	30.6	2.95	0.64	0.080	0.7	1.0
50	50.0	1.91	0.64	0.054	1.8	1.8
70	69.1	1.83	0.64	0.061	2.6	3.1
100	100.0	—	0.64	0.071	—	5.9

Key: S, sedimentation coefficient $S_{20,w}^0 \times 10^{13}$ cm/sec; D, diffusion coefficient $D_{20,w}^0 \times 10^7$ cm²/sec; \bar{V} , partial specific volume; η , viscosity cm/dl; MW, molecular weight $\times 10^{-6}$.

^a From Tissières *et al.* (1959).

^b Calculated from S and D.

^c Calculated from S and η .

Electron microscopy of purified ribosome preparations provides the dimensions shown in Table IV (Hall and Slayter, 1959; Huxley and Zubay, 1960).

Electron microscopy of sectioned bacteria is more ambiguous. Some sections show dense regions which may be due to ribosomes, others which might be expected to show ribosomes do not. It is not at all clear whether these differences are due to differences in staining techniques or whether the ribosomes do not exist as compact spheres in the living cell (Hanson *et al.*, 1959).

TABLE IV
DIMENSIONS OF *E. coli* RIBOSOMES

Particle	Shape	Dimensions (Å)	Mol. wt. $\times 10^{-6}$
30S	Prolate	95 \times 170	0.76
50S	Oblate	170 \times 140	2.0
70S	Oblate	200 \times 170	3.4

The protein/nucleic acid ratio of 37/63 corresponds closely to a ratio of 2 amino acids/nucleotide. Furthermore, a portion of the protein can be removed (see Section III,A) leaving another portion still in the ribosome structure. Electron microscopy of ribosomes stained with uranyl acetate shows no evidence of a protein shell around a nucleic acid core (Huxley and Zubay, 1960). These findings suggest that the 2/1 ratio may not be fortuitous but a consequence of saturating the entire RNA strand with protein along its entire length.

The hyperchromicity of ribosomes is 40%, the same as that of the RNA after isolation from the ribosomes. Thus, some aspects of the RNA structure are not appreciably altered by the addition of protein (Schles-

singer, 1960). A similar conclusion is drawn from X-ray diffraction studies (Zubay and Wilkins, 1960).

III. FRACTIONATION OF RIBOSOMES

A. CHROMATOGRAPHY

Chromatography on DEAE cellulose columns provides a means for separating protein, S-RNA, ribosomal RNA, and DNA from ribosomes. It is particularly useful in distinguishing the precursors in ribosome synthesis.

The column ($1 \text{ cm}^2 \times 10 \text{ cm}$) is prepared by packing a slurry of DEAE, thoroughly washed with tris buffer (0.01 M , pH 7.6, 0.01 M Mg), at 3–5 psi pressure. The preparation to be analyzed (whole cell juice or ribosome pellet) is adsorbed on the column from this buffer and eluted by 200 ml of the buffer in which there is a linearly increasing concentration of NaCl ($0\text{--}1 \text{ M}$).

Under these conditions ribosomes elute at 0.4 M NaCl, S-RNA at 0.5 M , and DNA at 0.6 M . Ribosomal RNA (prepared by phenol) remains adsorbed. The ribosomes emerge as 30 and 50S particles with their full complement of protein. If the magnesium concentration is

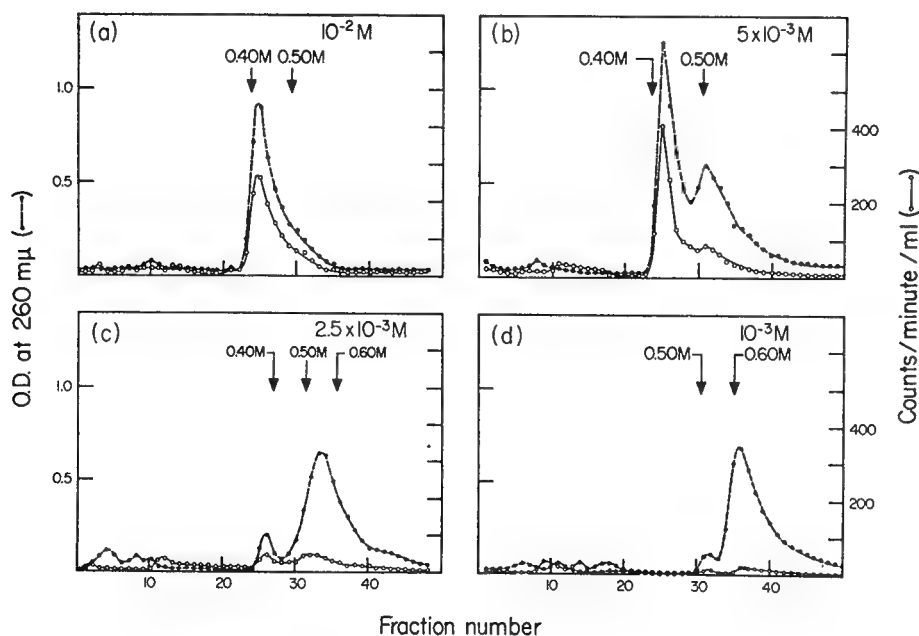


FIG. 9. Chromatography on DEAE of 70S ribosomes using different concentrations of magnesium. Protein content is indicated by S^{35} radioactivity.

decreased the ribosomes lose protein and elute at higher salt concentrations, as shown in Fig. 9.

The use of the DEAE column to separate ribosomes and their precursors for kinetic analysis was first reported in 1957 (Roberts *et al.*, 1958). Recent examples of this technique are given in Section V,A. Separation is achieved because of the different protein content of the ribosome and precursors.

B. SEDIMENTATION ANALYSIS

The separation of ribosomes of different sedimentation coefficients can be carried out by sedimenting a layer of ribosome suspension through a solution stabilized by a density gradient (Brakke, 1953; Britten and

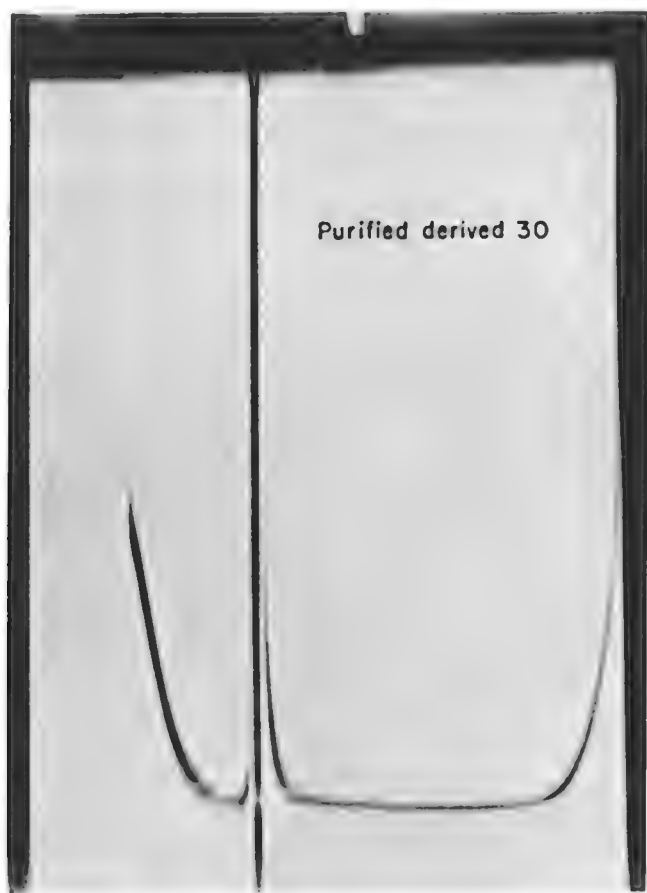


FIG. 10. Analytical centrifuge pattern of 30S ribosomes purified by centrifugation through a sucrose density gradient.

Roberts, 1960). Figure 10 shows the purity of 30S ribosomes prepared in this way. When small quantities of ribosomes are used in very thin layers excellent resolution can be achieved, as shown in Section V,B below.

C. DENSITY GRADIENT BANDING

Since DNA, RNA, and ribosomes have different densities they can be separated by prolonged centrifugation in appropriate solutions of high density. The materials form bands at the levels where their density equals the density of the solution. Materials differing by only a few per cent in density can be well separated since the density of the solution varies only slightly from top to bottom of the centrifuge tube. Density banding was used to detect the DNA-RNA hybrid complex (Hall and Spiegelman, 1961; Spiegelman *et al.*, 1961) and to show an association between DNA and ribosomes (Nisman, 1961).

Density banding also provides a unique method of separating newly formed ribosomes from old ones. The cells are first grown in media containing heavy isotopes (C^{13} , N^{15} , and H^2) and then transferred to media containing light isotopes. The ribosomes formed before and after the transfer can then be separated by density gradient banding. The value of this technique is illustrated in the work of Brenner *et al.* in their studies of phage-infected cells (Brenner *et al.*, 1961).

D. ELECTROPHORESIS

Electrophoresis has been used occasionally in the study of ribosomes. Pardee *et al.* showed a difference in the electrophoretic pattern caused by growth of the cells in chloramphenicol (Pardee *et al.*, 1957). Electrophoresis was used to isolate the RNA formed after phage infection (Nomura *et al.*, 1960).

E. TWO-PHASE SYSTEMS

Successful separation of ribosomes from other cellular components by partition in two-phase systems is described by Albertsson (1960). This method has not been exploited extensively in the study of ribosomes but it gives promise of being extremely useful in the search for complexes of ribosomes with DNA or for other studies of cellular organization.

F. CONCLUSION

There is still no general technique for distinguishing ribosomes of slightly different composition. Hence there is no method for determining whether there are two or a thousand different types of ribosomes. The only technique applied to date is the specific precipitation of the ribo-

somes which carry a particular protein by the antibody to that protein. Ribosomes have been precipitated by specific antibodies (Cowie *et al.*, 1961; Warren and Goldthwait, 1961) but analysis shows only a marginal difference in base composition from the average (Midgley and Goldthwait, 1961).

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B. Kinetic Studies of the Synthesis of RNA and Ribosomes

III.B.1 The Synthesis of Ribosomes in *E. coli*, 2, Analysis of the Kinetics of Tracer Incorporation in Growing Cells

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ABSTRACT Equations are derived representing the flow of radioactive tracer in a sequence of reactions. The conditions under which the equations are applicable are defined. A function ϕ , representing the newly synthesized fraction is defined, and its use in the analysis of precursor-product relationships is discussed.

A. INTRODUCTION

One of the great advantages of radioactive tracers in biological experimentation is the possibility of directly demonstrating in a complex system, for example a growing cell, that a particular sequence of reactions occurs. A radioactive compound may be chosen which enters into only a limited set of reactions; thus only a small number of cellular components become radioactive. These components may be separated from each other by much simpler procedures than those required for complete purification. By measuring the rate of entry of radioactivity into these intermediates and into the final products, proof of a sequential relationship can be obtained.

This paper examines the assumptions underlying such a proof, and presents a convenient method for analysis. The specific purpose is to introduce in a coherent form the arguments and method utilized in the following paper which reports studies of the stages of synthesis of ribosomal particles. Therefore in this discussion, although the analysis is entirely general, complicated and vague expressions have been avoided by referring almost entirely to the synthesis of RNA.

B. DERIVATION OF EQUATIONS REPRESENTING FLOW OF TRACER

1. *Restrictive Conditions.* In order to derive relatively simple equations representing the time course of passage of radioactivity through a sequence of intermediate objects, a number of severely restrictive conditions must be met.

(a) The relative quantity of intermediates should not change with time. This

statement is equivalent to a requirement for steady exponential growth of the culture.

(b) From a mathematical point of view each cell should obey condition (a) and the population should be homogeneous. However this is not achievable since growth normally occurs as a result of replication, followed by cell division. It will be presumed that the cells in the population are randomly phased and that any effects of variation of processes with time during the division cycle will be lost, since the samples of the population analyzed are always random.

(c) The specific radioactivity and rate of utilization of the tracer must be constant.

(d) It will also be assumed that the reactions which cause the transfer of tracer from one intermediate state to another are unidirectional. The failure of this condition does not necessarily lead to greater complexity, but such a case must be separately considered.

(e) Finally, it will be presumed that any molecule representative of a particular intermediate stage has a constant chance of being transferred to the next stage regardless of the length of time it has been present.

In a given experiment the proof that these conditions are actually met may not be available, or it may be known that one or more of them fail. In such a case careful analysis must be carried out in order to evaluate the type and magnitude of error that may result.

If the first precursor to be considered happens to be a pool of low molecular weight intermediates, it will most often be true that the specific radioactivity of the pool will rise with time as $1 - e^{-at}$. If there is no exchange between the external tracer and unlabeled pool compounds, the equations given in sections B and C will be valid as they stand. If exchange occurs, the equation for ϕ_T will not be valid and the time constant for the first precursor will be shorter than that expected from the size of the precursor pool. If this is taken into account, the remaining equations are still useful.

2. Definition of Symbols.

τ	Time after addition of tracer; units such that $\tau = 1$ when the cells have grown by a factor e ; $Q = Q_0 e^\tau$ gives the growth of the cells or any component
μ	Effective specific radioactivity of tracer; units such that the specific radioactivity of the RNA will approach μ after a long period of growth at a constant tracer concentration
X	Quantity of a component, in general
X^*	Its radioactivity
μ_x	Its specific radioactivity
T	The total of all components
M, N	The quantities of RNA in the precursor and product, section 3

$$\phi_x = \frac{\mu_x}{\mu} \frac{X}{T} = \frac{X^*}{\mu T}$$

3. *Calculation of the Time Course of Labeling of a Single Precursor and Product.* From the steady exponential growth of each component:

$$M = M_0 e^{\tau}, \quad N = N_0 e^{\tau}, \quad T = T_0 e^{\tau}$$

The total RNA synthesized after $\tau = 0$ is:

$$T - T_0 = T_0(e^{\tau} - 1)$$

The newly synthesized fraction of the total RNA:

$$\phi_T = \frac{T_0(e^{\tau} - 1)}{T_0 e^{\tau}} = 1 - e^{-\tau} \quad (1)$$

The rate of change of the radioactivity of the precursor, M :

$$\frac{dM^*}{d\tau} = \mu \frac{dT}{d\tau} - \mu_M \frac{dN}{d\tau} = \mu T - \mu_M N$$

and

$$\frac{dM^*}{d\tau} = \frac{d}{d\tau} (\mu_M M) = \mu_M M + M \frac{d\mu_M}{d\tau}$$

therefore since $T = M + N$:

$$\frac{d\mu_M}{d\tau} = \frac{T}{M} (\mu - \mu_M)$$

Integrating and using the condition that $\mu_M = 0$ when $\tau = 0$:

$$\mu_M = \mu(1 - e^{-(T/M)\tau})$$

The newly synthesized fraction of the total RNA which is present in the precursor:

$$\phi_M = \frac{M}{T} \frac{\mu_M}{\mu} = \frac{M}{T} (1 - e^{-(T/M)\tau}) \quad (2)$$

Finally, since $\phi_T = \phi_M + \phi_N$ the newly synthesized fraction of the total RNA which is present in the product:

$$\phi_N = 1 - e^{-\tau} - \frac{M}{T} (1 - e^{-(T/M)\tau}) \quad (3)$$

Fig. 1 shows the three functions ϕ_T , ϕ_M , and ϕ_N calculated from equations (1), (2), and (3) for the specific case where $M/T = 0.10$. The interpretation of the log-log plot will be discussed below; however, in this mathematical section will be given the proof that ϕ_M and ϕ_N have the form at early times indicated on Fig. 1.

Expansion of the exponential gives for equation (2):

$$\frac{M}{T} \left(1 - 1 + \frac{T}{M} \tau - \frac{T^2}{2M^2} \tau^2 \dots \right) = \tau - \frac{T}{2M} \tau^2 \dots$$

At early times when $T\tau/2M \ll 1$, $\phi_M = \tau$ and ϕ_M thus follows a straight line at 45° (slope 1) on Fig. 1.

Similarly, expansion of the exponentials in equation (3) gives:

$$\tau - \frac{\tau^2}{2} + \frac{\tau^3}{6} \cdots = \tau + \frac{T}{M} \frac{\tau^2}{2} - \frac{T^2}{M^2} \frac{\tau^3}{6} \cdots$$

Here the terms in τ cancel at early times and

$$\phi_N = \frac{N\tau^2}{2M} \left(1 - \frac{T + M}{3M} \tau \cdots \right). \quad (4)$$

ϕ_N has the form indicated on Fig. 1 at early times and as a matter of fact deviates from this by less than 10 per cent when $\tau = .03$. At this time the precursor has already reached one-third of its final radioactivity. Thus for a usefully long period the radioactivity of the product rises as τ^2 and follows a straight line of slope 2 on the log-log plot.

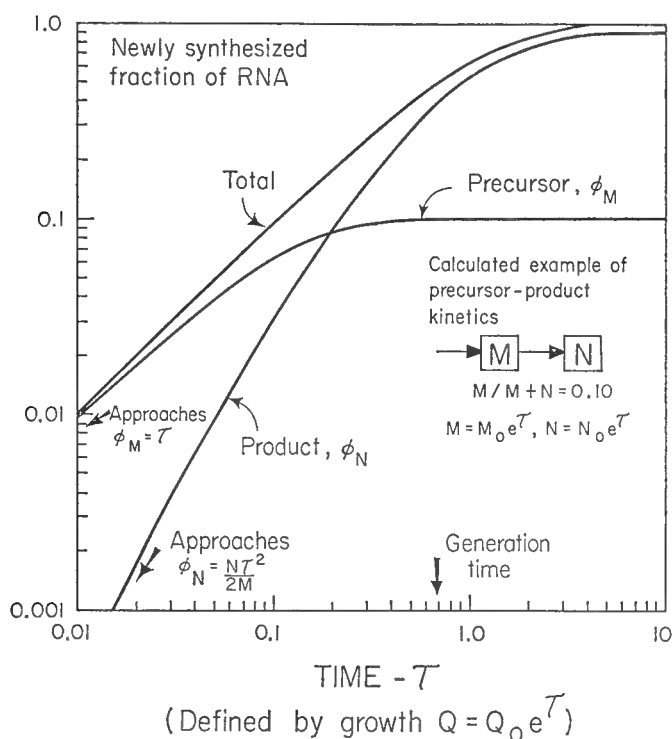


FIGURE 1 Example of precursor-product relationship. Calculated from equations (1), (2), and (3) for the case in which one tenth of the total RNA is in the precursor. The precursor levels off at 0.10 and reaches 63 per cent of this value when $\tau = 0.10$. Ordinate and abscissa scales defined in text.

4. *The Time Course of Labeling of Two Sequential Precursors and a Product.* Where the tracer enters sequentially into 3 objects ($\rightarrow E \rightarrow N \rightarrow R$) an analysis similar to that given in section 3 but involving one further integration yields the following equations:

$$\phi_T = 1 - e^{-\tau} \quad (5)$$

$$\phi_E = \frac{E}{T} (1 - e^{-(T/E)\tau}) \quad (6)$$

$$\phi_N = \frac{N}{T} \left(1 - \frac{E(R + N)e^{-(T/E)\tau} - TNe^{-(R/N)\tau}}{E(R + N) - TN} \right) \quad (7)$$

$$\phi_R = \phi_T - \phi_E - \phi_N \quad (8)$$

Analysis of the time course of labeling of an indefinite number of such sequential precursors may be easily carried out in a similar way. At early times the radioactivity of each of the objects in sequence will rise in proportion to τ , τ^2 , τ^3 , τ^4 , etc. Without a detailed study of the approximations involved this may be easily seen from the following argument.

At early times the first precursor will contain essentially all of the radioactivity and its specific radioactivity will rise in proportion to τ . Since the amount of radioactivity in the second precursor is simply the time integral of the specific radioactivity of the first, it will rise in proportion to τ^2 , as long as the specific radioactivity of the first does not approach saturation. Similarly the amount of radioactivity in the third object is the time integral of the specific radioactivity of the second and will rise to proportion to τ^3 .

C. DISCUSSION

The function ϕ which we have called the newly synthesized fraction of the RNA (in the special case of RNA synthesis) has turned out to be of great practical use. It can be expressed:

$$\phi_X = \frac{\mu_X}{\mu} \frac{X}{T} = \frac{X^*}{\mu T} \quad (9)$$

It is measured by the radioactivity (X^*) of a given fraction, and can be computed readily from the experimental data. At early times the total radioactivity of a precursor may be measured accurately even when the specific radioactivity is uncertain as a result of dilution with unlabeled material. T is, of course, the total RNA corresponding to the sample size on which X^* was determined.

μ is readily determined from the specific radioactivity of the total RNA using the relations:

$$\mu_T = \frac{T^*}{T} = \mu\phi_T = \mu(1 - e^{-\tau}) \quad (10)$$

where τ has been determined from the growth curve, $Q = Q_0 e^\tau$. Since T is used both in calculation of μ and ϕ_X an absolute measure of RNA is unnecessary. In fact a number proportional to the equivalent cell mass in the sample is sufficient.

In using equation (10) it is, of course, necessary to know that T^* is in fact the total radioactivity in the sequence of reactions being considered. Further if T^*/T is

not proportional to $1 - e^{-\tau}$ a failure of restrictive condition *c* or *d* is indicated and the significance of the errors resulting must be considered. Examples of the method of calculation of ϕ and its use in a complex case are given in Paper III.

In the interpretation it is convenient to plot $\log \phi$ against $\log \tau$. This procedure expands both scales at early times. During the usefully long period when ϕ is proportional to τ , τ^2 , or τ^3 a straight line of slope 1, 2, or 3 results. Thus precursor-product relationships can be readily recognized. The relative quantity of each fraction is given by the final value of ϕ after the specific activity of that fraction has been saturated.

In a simple case such as that of a single precursor it is easy to test the precursor-product relationship quantitatively without further calculation. In this case ϕ for the precursor is given by equation (2). On the log-log plot the constants in equation (2) affect the position but not the shape of the curve. Thus a curve of $1 - e^{-\tau}$ on tracing paper may be translated over the experimental plot until the best fit is achieved. The experimental points, at late times, should level off at $\phi = M/T$. $\tau = 1$ on the $1 - e^{-\tau}$ tracing should lie over $\tau = M/T$ on the experimental graph.

In more complex cases, or if there is a failure of a sequential relationship, the nature of the relationships can be deduced from the slopes, positions, and curvatures of the various fractions. The use of τ defined by the growth curve simplifies all of these operations and allows the comparison of experiments carried out at different growth rates.

While ϕ is adequately defined by equation (9) the following general definition may perhaps be useful. ϕ is the ratio of the number of labeled atoms at a given time in a given class of molecules to the number of atoms in the whole cell which would be labeled after a long period of growth during which the labeling conditions were precisely constant. ϕ is thus a measure of the number of newly synthesized molecules which occur in a given class, and has been named the "newly synthesized fraction." The term newly synthesized is used here to refer to those molecules which have actually been assembled from low molecular weight precursors (including the tracer) after the tracer has been added. If over a significant period of time there is a rise in the specific radioactivity of a pool of low molecular weight precursors, the general definition above is still valid but the meaning of the term newly synthesized must be carefully considered.

APPENDIX

A SPECIAL METHOD USING SPECIFIC RADIOACTIVITIES

In certain circumstances the analytical methods permit the measurement of the specific radioactivity of the fractions, but not their total radioactivity. In such cases the equations given here are still useful. However, in the absence of independent measures of the time constant and the size of a fraction the test of precursor-product relationships is not as effective. The purpose of this Appendix is not to examine such procedures but to

bring attention to the peculiar properties of the function μ_N/μ_M , the ratio of the specific radioactivity of the product to that of the precursor in the simple case examined in section 3. From equations (2) and (3) we can write:

$$\frac{\mu_N}{\mu_M} = \frac{M}{N} \left(\frac{T}{M} \cdot \frac{1 - e^{-\tau}}{1 - e^{-(T/M)\tau}} - 1 \right) \quad (11)$$

At early times this ratio has, of course, very small values, and at late times it approaches unity. However, at any given time it does not vary by more than a factor of two for any possible relative quantities of precursor and product. For example, when $\tau = 0.01$, $\mu_N/\mu_M = 0.01$ for the case of an infinitely small precursor. For the other extreme case when the precursor is large and the product infinitely small $\mu_N/\mu_M = 0.005$ when $\tau = 0.01$.

This surprising result is not evident by inspection of the equation. It should be useful in a variety of special circumstances. For example, incomplete purification of the precursor will cause the experimental curve to lie entirely outside of the region permitted by this function. This can easily be recognized and does not require knowledge of the relative size of the pool of precursor. The existence of two sequential precursors or the failure of restrictive condition (*e*) will yield values of μ_N/μ_M much smaller than those predicted by equation (11).

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Comment. Another example of the use of this type of analysis is provided by the paper of Rake and Graham (*Biophys. J.*, in press, 1964). Sequential stages in the synthesis of RNA in tissue cultures of mouse cells are elucidated by analysis of the kinetics of incorporation of C¹⁴-adenine. Brian J. McCarthy.

III.B.2 The Synthesis of Ribosomes in E. coli, 3, Synthesis of Ribosomal RNA

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ABSTRACT Techniques of chromatography on columns of DEAE¹ cellulose and sedimentation analysis through a sucrose gradient have been used to study the flow of C¹⁴-uracil label through precursors to completed ribosomes. Analysis by chromatography shows the existence of two sequential precursors constituting together some 10 per cent of the total ribosomal RNA. The chromatographic separation into three fractions is ascribed to the lower protein/RNA ratios of the precursor. By sedimentation the primary precursor (eosome) is identified as a component of average sedimentation coefficient 14S. The second precursor stage (neosome) is divided among at least two particles, one of 43S and the other of about 30S. Detailed kinetic analysis shows that all the radioactivity passes through the eosome on its way to finished 50S and 30S ribosomes. The delay in the entry of radioactivity to ribosomes is that expected from the quantity of eosome precursor. The obvious conclusion that there exists a precursor-product relationship is discussed together with possible interpretations.

A. INTRODUCTION

A number of experiments have shown that ribosomes serve as the principal sites of protein synthesis in living cells (McQuillen, Roberts and Britten, 1959). In addition, ribosomes are an essential ingredient for the low but probably significant rate of peptide bond synthesis which occurs in cell-free systems (Tissières *et al.*, 1960). In the larger cells, the ribosomes appear capable of protein synthesis in regions remote from DNA. Thus the information needed to arrange the amino acids in proper order must reside in the material of the ribosome or in a part of that material. Accordingly, a study of the biosynthesis of ribosomes might reveal not only the assembly of an important part of the cells' synthetic machinery but also indicate the mechanism by which the information of the DNA directs the synthesis of specific proteins.

Our studies of ribosome synthesis have continued since 1957. Progress in our understanding of the process has been reported annually (Roberts *et al.*, 1958,

¹ DEAE= diethylaminoethyl

1959, 1960) and at various meetings (Roberts, 1960). In addition some particular aspects of the work have been published (Roberts, Britten, and Bolton, 1958; McCarthy and Aronson, 1961). The earliest work (Roberts *et al.*, 1958) showed clearly that tracer material, en route to mature ribosomes, passed through several precursor stages. These were distinguished by differences in their chromatography and in their sedimentation. In 1958 it was estimated that roughly 10 per cent of the RNA was found in the precursor stage as nucleic acid or nucleoprotein of low protein content.

Several changes in technique have recently been introduced which make significant improvements in the accuracy of the measurements and in the possibility of interpretation. In the early experiments there was some degradation of ribosomes during chromatography on DEAE cellulose. This degraded material contaminated the precursor region and obscured the kinetics. This difficulty has been reduced by the use of a higher magnesium concentration in the eluting fluid. The accuracy of the measurements has been improved throughout by using P^{32} as a measure of total RNA together with C^{14} -uracil to indicate newly formed RNA.

This technique also permits the use of much smaller quantities of material so that much higher resolution can be obtained in sedimentation analysis. In particular, sedimentation analysis carried out in low concentrations of magnesium (10^{-4} M) has greatly aided the interpretation of all the results. Under these conditions two precursor stages are separable from the product material. In high magnesium concentrations the first precursor is associated with ribosomes of all sizes and its properties are obscured.

Recently another type of RNA has been postulated, "Messenger RNA" (Jacob and Monod, 1961). This material is presumed to have a nucleotide composition similar to that of DNA and to have a high rate of turnover, being used to carry information from DNA to the site of protein synthesis and then being degraded to nucleotide. Evidence for the existence of RNA having complementarity to DNA has been found by Hall and Spiegelman (1961) who showed that hybrid molecules could be formed between some RNA and its related DNA.

Other studies (Gros *et al.*, 1961) directed towards the detection of messenger RNA have reported the presence of an RNA fraction which is rapidly labeled. However the properties of this fraction are indistinguishable from the properties of the ribosome precursor. Thus the question arises whether in addition there is a small fraction of rapidly turning over RNA which has a nucleotide sequence complementary to DNA, or whether the ribosome precursor may itself carry out the function of the postulated messenger.

In this and a following paper we present the results of studies of the incorporation of P^{32} and C^{14} -uracil into the RNA portion of ribosomes and the incorporation of C^{14} -amino acids into the protein portion. In addition ways of distinguishing ribosome precursor from messenger RNA are discussed.

B. MATERIALS AND METHODS

1. *Growth of Cells.* *E. coli* ML 30 was grown at 37° in C medium (Roberts *et al.*, 1955) with maltose as carbon source. An overnight culture was diluted with fresh medium and growth followed by optical density measurements at 650 m μ , for two or three generations of exponential growth so that an accurate estimate of the growth rate could be made under steady-state conditions. The generation time did not vary significantly from 51 minutes in any of these experiments.

Kinetic experiments began with the addition of the tracer. Measurements of the uptake of tracer into macromolecules (Paper I) and the increase in optical density were continued throughout the experiment.

In many experiments it proved convenient to use tracer methods for the measurement of total RNA as well as newly synthesized RNA. In these cases cells were grown for about three generations in C medium containing 1 to 2 mc P³²O₄ per liter so that at least 88 per cent of the RNA molecules were P³²-labeled. The steady-state P³²-labeling of the RNA amounting to 70,000 to 140,000 cpm per mg RNA was used as a sensitive and accurate measure of the total nucleic acid in fractions free of phospholipid.

2. *Estimation of Radioactivity.* The 2-C¹⁴-uracil was obtained from the California Corporation for Biochemical Research and the New England Nuclear Corporation and had specific radioactivities in the range 4 to 10 mc/mm.

Radioactivity present in RNA was estimated by adding trichloroacetic acid (TCA) to 5 per cent and passing through a 1 inch millipore filter (Britten, Roberts, and French, 1955). The filter was air-dried at 60-70°C for half an hour and suspended upright in a vial containing 10 ml of a 2,5-diphenyl oxazole 4 gm/liter (PPO) and 1,4-bis-2-(5-phenyl-oxazolyl) benzene 100 mg/liter (POPOP) solution in toluene. The samples were counted in an automatic Packard tri-carb liquid scintillation counter. Simultaneous counting of P³² and C¹⁴ was achieved with the aid of the split channel setting. At 870 volts C¹⁴ was counted with 40 per cent efficiency in the 10 to 50 volt channel with P³² contamination of 5 per cent. P³² was counted in the 100 volt to infinity channel together with only 1 per cent of the C¹⁴ count. The magnitude of these corrections was sufficiently low and constant for them to be made reliably on a routine basis.

3. *Analysis of Cell Extracts.* Cell samples were taken at suitable times during kinetic experiments by pouring rapidly onto crushed frozen medium. The cells were washed three times with cold tris chloride buffer 10⁻² M, pH 7.4 containing magnesium chloride at concentrations from 10⁻² to 10⁻⁴ as specified. The cells were resuspended in 1 to 2 ml of buffer and an extract prepared by passing through the orifice of a French pressure cell at 10,000 to 15,000 psi. In many cases DNAase was added to the cells immediately prior to the preparation of an extract.

Analysis of the cell extract on the basis of the varying rates of sedimentation of the various components, "sedimentation analysis," was made by the sucrose gradient technique already described (Britten and Roberts, 1960) with modifications to obtain higher resolution. With the original technique, the lower limit of the load of cell extract was determined by the necessity to read the ultraviolet absorption of the fractions. This normally required grading a 0.2 ml sample on the top of the swinging bucket tube. The use of a steady-state P³² label for measurement of the quantity of RNA greatly improved the sensitivity and made possible the use of much smaller samples of cell extract. Small samples are most conveniently loaded on the sucrose gradient by means of a pipet. A 0.07 or 0.05 ml drop of the cell extract was placed on a silicone-treated microscope slide near another drop of equal volume of 4 per cent sucrose solution in a tris buffer

containing MgCl_2 at a concentration from 10^{-2} to 10^{-4} M. A 0.2 ml pipet having the tip drawn out and slightly bent was used to suck up first the cell extract sample and then the sucrose solution. Slight tipping of the pipet removed any sharp boundary which might have formed between the two solutions. The whole contents of the pipet was then allowed to run gently onto the top of a 4.8 ml linear sucrose gradient from 20 per cent to 5 per cent in the same buffer made in the normal manner. After centrifuging for an appropriate time the contents of the tube was dripped out into about fifty fractions.

After adding 2 ml of cold 5 per cent TCA and allowing to stand for 20 minutes the radioactivity present in macromolecules was collected by passing through a membrane filter.

The DEAE cellulose used for chromatography of cell extracts was obtained from the California Corporation for Biochemical Research (cellex D, 0.91 meq/gm). The material was suspended in tris buffer 0.01 M containing MgCl_2 0.01 M and brought to pH 7.4 with HCl. After the coarse fraction settled, the fine material was decanted with the supernatant. Three such washes were given. Columns of about 15 cm were packed in 1 cm tubes under 3 psi pressure. The samples of cell extract or ribosomes analyzed contained a maximum of about 5 mg RNA.

Elution was carried out with a linear sodium chloride gradient from zero to 1.0 M or from 0.2 M to 1.2 M in tris buffer 0.01 M, pH 7.4 containing MgCl_2 0.01 M. The salt gradient was about 0.004 M/ml and the flow rate about 0.5 ml/min. About fifty fractions of 3.5 to 4.0 ml were collected.

4. *Extraction of RNA.* Duponol at 0.5 per cent was added to the total cell extract. This treatment dissociates the RNA from the protein (Kurland, 1960). Ribonuclease activity was prevented by the addition of 0.5 per cent duponol to the sucrose solutions in tris-HCl 0.01 M pH 7.4 used in sedimentation analysis.

C. RESOLUTION OF NUCLEOPROTEIN ON DEAE-CELLULOSE

Chromatography of a total cell extract on DEAE cellulose provides a useful method of separating ribosomes from the bulk of soluble cellular protein. While most of the soluble protein is eluted between zero and 0.3 M NaCl, ribosomes give a single peak at 0.4 M. Behind this ribosome peak, S-RNA elutes at 0.5 M and DNA at 0.6 M. Chromatography of P^{32} pulse-labeled extracts on DEAE shows the radioactivity in a region resolved from the main bulk of the ribosomes close to those of S-RNA and DNA. (Roberts *et al.*, 1958). At later times this radioactivity moves into the main nucleoprotein peak. These analyses suggested the existence of two sequential precursors in the synthesis of the RNA of the main nucleoprotein peak and that the resolution was a result of the lower protein content of the precursors. Detailed kinetic analysis of the results was not attempted due to the delay of the entry of the P^{32} radioactivity used as tracer into RNA by the large pool of nucleotide precursors. In contrast the special features of the entry of C^{14} -uracil into RNA already discussed (Paper I), giving an essentially linear curve of incorporation from time zero, make it much more suitable.

In Fig. 1 the DEAE chromatographic profiles are shown for four samples of a total cell extract taken 10, 20, 40, and 55 minutes after the addition of C^{14} -uracil.

The 0.4 M peak contains most of the ribonucleoprotein and the 0.5 to 0.6 M region S-RNA and the precursors. Even at 10 minutes it is evident that the precursor region has a specific radioactivity three to five times that of the main peak. Between 10 and 20 minutes the specific radioactivity of the main peak rises by more than a factor of three, demonstrating directly the existence of a sizeable precursor pool preceding it (Paper II).

A second experiment in which shorter exposures to C^{14} -uracil were employed

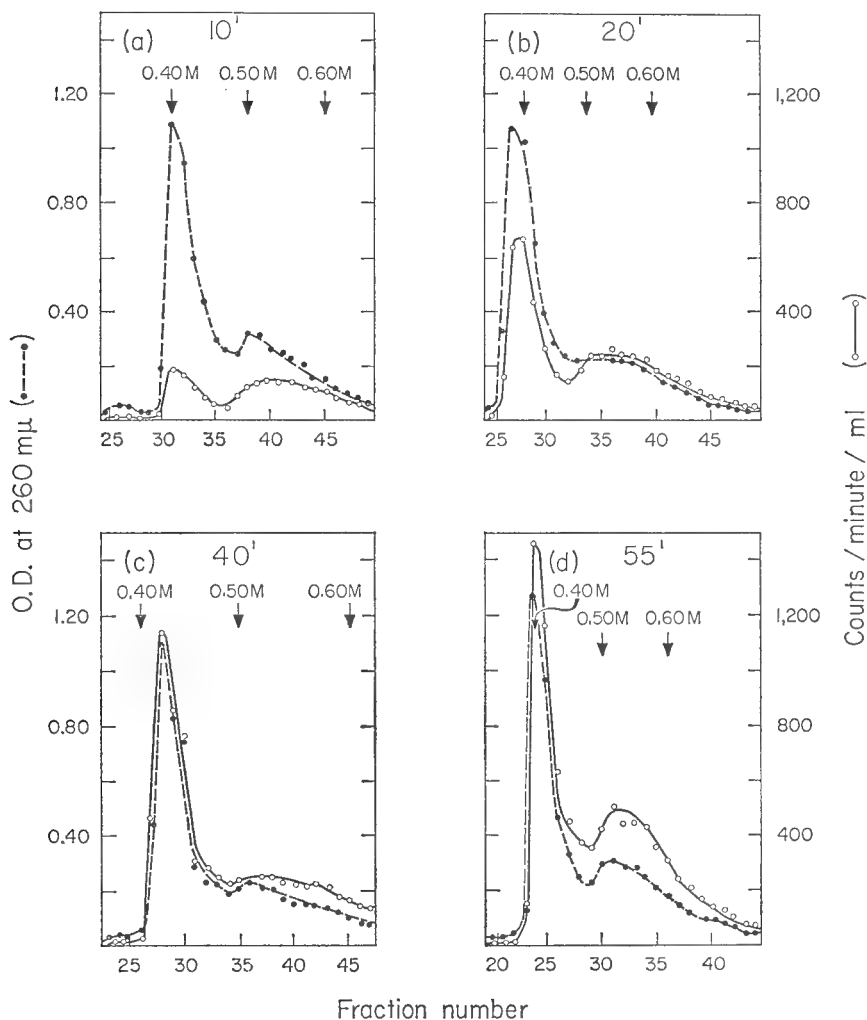


FIGURE 1 Analysis of total cell extracts on a DEAE-cellulose column. Linear sodium chloride gradient from zero to 1.0 M tris-HCl buffer 0.01 M, containing $MgCl_2$ 0.01M, pH 7.4, salt gradient 0.004 M/ml. Only the region in which RNA appears is plotted. The volumes collected varied between the four analyses but salt concentrations are indicated on each. The main peak includes most of the ribosomal material. S-RNA peaks at 0.5 M. (a) 10 minute exposure to C^{14} -uracil, (b) 20 minute, (c) 40 minute, (d) 55 minute.

proved to be more suitable for the separation of the three stages of ribosome synthesis and for the elucidation of their quantities and the flows through them. Six samples of cells were taken from a culture given from 25 second to 12 minute exposure to C^{14} -uracil. The cell extracts in tris buffer 10^{-2} M Mg^{++} were centrifuged for 4 hours at 40,000 RPM. The pellet contained essentially all of the ribosomal material and very little S-RNA. Chromatography of this pellet eliminated contamination of the ribosome precursor region around 0.5 M salt by S-RNA (Fig. 2).

The sequence of ribosome synthesis is readily observed in the six parts of Fig. 2. At 25 seconds, Fig. 2(a), all the radioactivity appears in a single peak at 0.6 M salt.

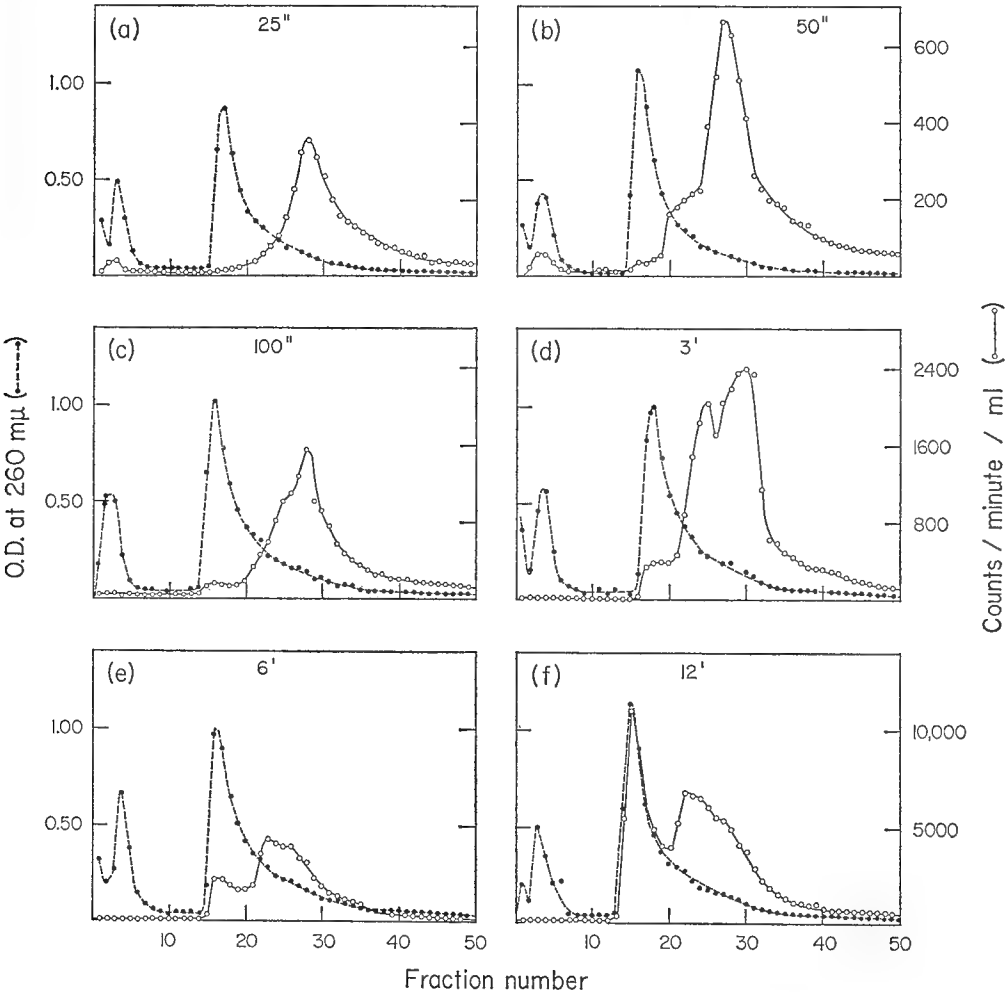


FIGURE 2 Analysis of total ribosomal pellets (40K 240 minute pellets) on a DEAE-cellulose column. Linear sodium chloride gradient from 0.2 M to 1.2 M in tris-HCl buffer 0.01 M containing $MgCl_2$ 0.01 M, pH 7.4. Salt gradient 0.004 M/ml. Volumes collected 3.6—3.8 ml. (a) 25 second exposure to C^{14} -uracil, (b) 50 second, (c) 100 second, (d) 3 minute, (e) 6 minute, (f) 12 minute.

At 50 seconds, Fig. 2(b), and 100 seconds, Fig. 2(c), a shoulder develops on the forward edge of this peak at 0.5 M to become dominant at 6 minutes, Fig. 2(e). Meanwhile the radioactivity associated with the main peak remains at a very low level in the first three analyses, subsequently rising very rapidly from 3 minutes to 12 minutes as shown in Fig. 2(d), (e), and (f).

For purposes of clarity and ease of discussion two sequential precursors in ribosome synthesis have been named eosome and neosome in order of synthesis. As will be shown later, these definitions do not rest only upon the distinctive chroma-

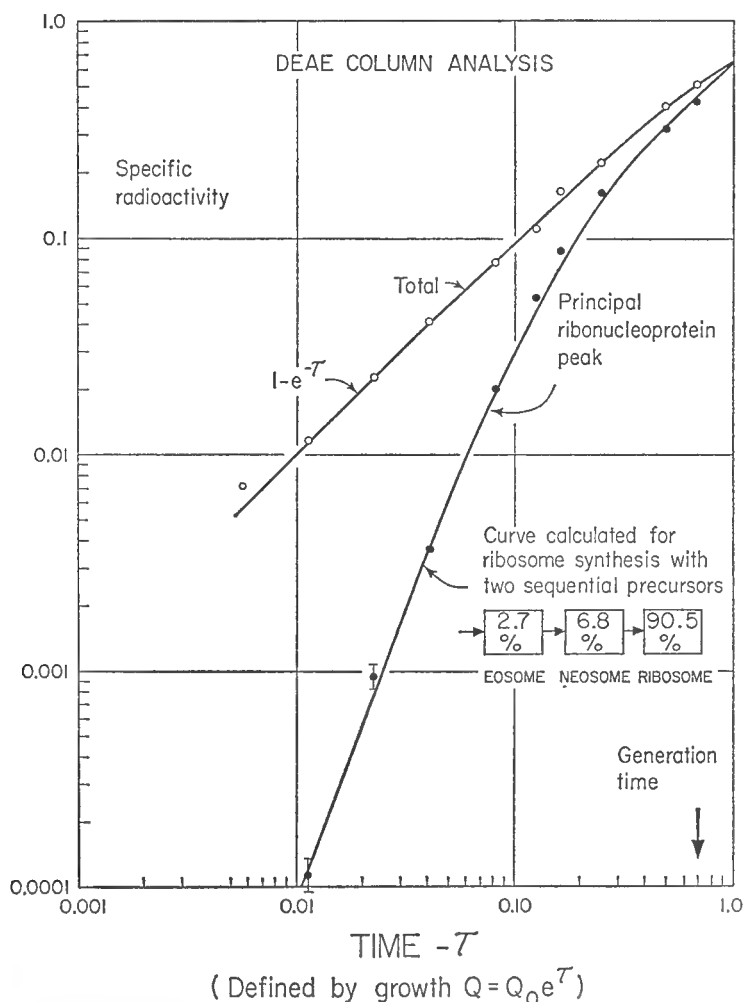


FIGURE 3 Log-log plot of the time course of specific radioactivity of the ribosome peak in a DEAE-cellulose column analysis. Time τ defined by $Q = Q_0 e^{\tau}$. Data from Fig. 1 and 2. The open circles represent the specific radioactivity of the total material eluted from the column $\Sigma C^{14} / \Sigma O.D., 260m\mu$. The curve drawn through them is $1 - e^{-\tau}$. The curve drawn through the solid circles representing ribosome specific radioactivity was calculated for the example indicated on the figure.

tographic behavior, for the eosome and neosome can also be resolved from the bulk ribosomal material on the basis of their sedimentation coefficients.

The detailed analysis of the distribution of radioactivity between the ribosome peak and the two precursor stages was made in the following way.

1. The sample times were converted to τ with the knowledge of the generation time, *i.e.*, $\tau = t \log_2 2 / \text{generation time}$.

2. The total uracil label and the ultraviolet absorption at 260 m μ were summed for each column analysis and the ratio $\Sigma C^{14} / \Sigma \text{O.D. } 260 \text{ m}\mu$ plotted against τ . Since this curve should fall on $\mu(1 - e^{-\tau})$, the factor μ was evaluated by sliding the points on the ordinate alone for a good fit (Fig. 3). The constant μ is a measure of the radioactivity per unit ultraviolet absorption of RNA at late times when $1 - e^{-\tau}$ approaches 1. It includes the specific radioactivity of the tracer and factors for the rate of utilization for RNA synthesis and the conversion to cytosine. The function is now $\phi_\tau = 1 - e^{-\tau}$ (Paper II). Since different concentrations and specific radioactivities of tracer were used μ was evaluated separately for the experiments of Figs. 1 and 2. Further, the slow rise in the rate of incorporation of uracil into RNA has been compensated in this way since the concentrations and time range of the two experiments were properly chosen.

3. The most easily calculated quantity is the specific radioactivity of the main ribosome peak μ_R / μ . Since it is the major component μ_R / μ is almost the same as ϕ_R . In this case it is in fact $1.1\phi_R$. No serious contamination of the first three or four fractions of this peak results from the radioactivity of precursor materials except in Figs. 2(a) and (b). No significant quantity of radioactivity can be attributed to the main ribosome peak in 2(a) and some correction for contamination was required for 2(b). The specific radioactivity is plotted against τ in Fig. 3. The magnitude of the depression of the ribosome specific radioactivity at early times is immediately diagnostic of two successive precursors since no one precursor could possibly be large enough to cause such a delay. The dependence of the specific radioactivity upon τ^3 at early times is also symptomatic of a delay by two precursors (Paper II). Fig. 3 also shows a theoretical curve drawn through the points, the justification for which will be given in a later paragraph.

4. A plot of ϕ_R versus Δ mass shows the delay of the entry of label into ribosomes due to the precursors. The intercept on the ordinate is a measure of the total quantity of precursor. This is true whether the precursors are sequential or parallel. Application of this procedure leads to a value of 9.5 per cent of the total ribosomal RNA as precursor.

5. Since the above determination indicates that the total quantity of precursor is much less than the total ultraviolet absorption in the region, it appears that most can be attributed to a streak of the main ribosomal peak. This can also be shown by chase experiments which fail to reduce the radioactivity in the region. Therefore, to a first approximation the radioactivity in the total precursor may be obtained by

subtracting the radioactivity due to the product ribosomal material at the specific radioactivity of the product peak. This is in fact a small correction except at very late times (Fig. 1). The total radioactivity in the precursor was then used to calculate ϕ_{N+E} which is plotted in Fig. 4.

6. Although it is apparent from the curves of Fig. 2 that there exist two components in the precursor region, the precision of any apportionment which can be made of the radioactivity between these two components is limited by the poor resolution. However from the earliest time point (25 seconds, Fig. 2(a)) a reason-

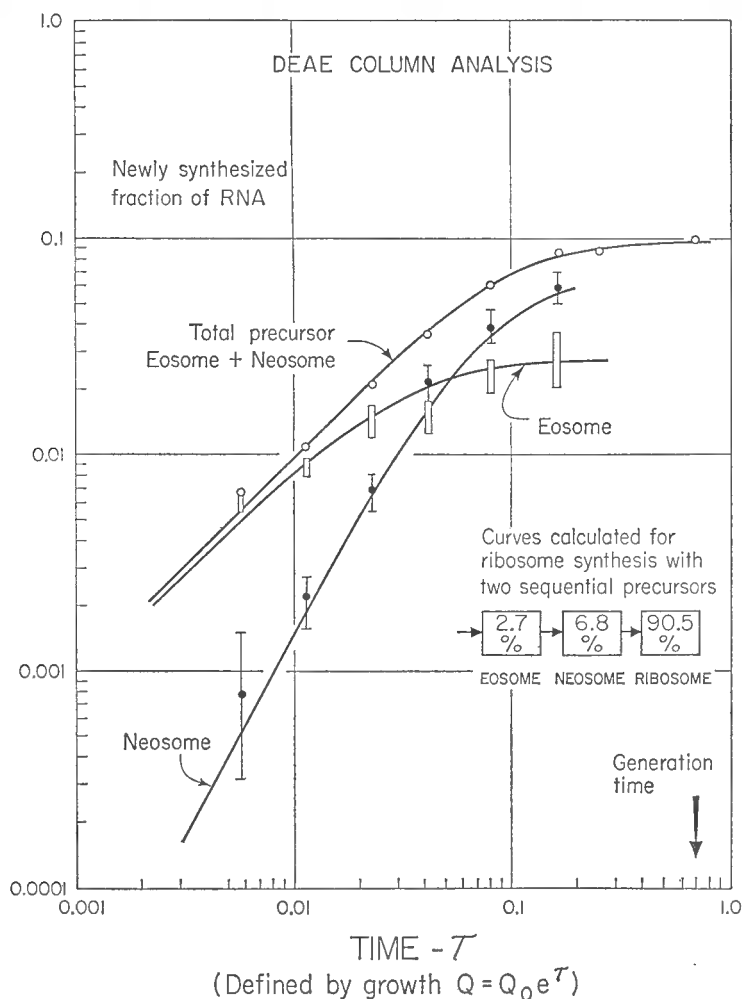


FIGURE 4 Log-log plot of the fraction of newly synthesized RNA present in total precursor and in eosome and neosome as a function of time. Data from Fig. 2 (two points from Fig. 1 at late times). The open circles represent total precursor, eosome plus neosome; the solid circles, neosome, and the bars eosome. The vertical lines are estimated ranges of possible error in the peak shape analysis. The three curves drawn are theoretical ones calculated for the example indicated on the figure.

able estimate of the peak shape of the eosome component may be made. Likewise, the peak shape of the neosome may be recognized from later points (Fig. 2(f)). The peak fractions of each of the components were located from the earliest and latest time points. The cross-contamination in each of these fractions by material of the other component was determined from the known peak shapes. Application of these corrections led to estimates of the fraction of the total precursor radioactivity attributable to each component. It was then possible to divide ϕ_{N+E} into ϕ_N and ϕ_E (Fig. 4).

7. To check the above procedure, numerical tables were made of functions for the peak shapes of the two precursor components. A least squares procedure was then applied to each time point for the separation into the two components. Agreement within the limits of precision was obtained between determinations made by the two procedures. In fact the evaluation of ϕ_N at very early times and ϕ_E at very late times, when each is a small fraction of the total precursor, is subject to considerable uncertainty due to the magnitude of the cross-contamination.

The results of these analyses are summarized in Figs. 3 and 4. The curve for μ_R/μ in Fig. 3 has a slope proportional to τ^3 at early times. Similarly the ϕ_N and ϕ_E curves of Fig. 4 have initial slopes proportional to τ^2 and τ . These relationships are direct indications of the sequence eosome \rightarrow neosome \rightarrow ribosome. The values at which the plots of ϕ_N and ϕ_E level off are direct measures of the quantities of each of the two precursors (Paper II). Of the total 10 per cent or so precursor material in ribosomal RNA about 2.5 per cent can be ascribed to eosomal material, and about 7 per cent to neosomal material. Theoretical curves for $\phi_N + \phi_E$ and μ_R/μ were calculated and fitted to the data in Figs. 3 and 4. The best fits were obtained using 2.7 per cent eosome, 6.8 per cent neosome, and 90.5 per cent ribosome.

D. INITIAL STUDIES BY MEANS OF SEDIMENTATION ANALYSIS

Most studies of the kinetics of ribosome synthesis have been carried out by means of sedimentation analysis in a magnesium concentration of 10^{-2} M necessary for the preservation of ribosomes. The general features of such analyses made on extracts from cells given a short pulse of either P^{32} or S^{35} have already been described (Roberts *et al.*, 1959; Roberts, 1960). At early times the specific radioactivities of the 30S and 50S ribosomes, present in the extract, were considerably higher than that of the 70S. These small particles, described as native 30S and 50S to differentiate them from those derived by breakdown of the 70S at lower magnesium concentrations, represent together some 10 to 20 per cent of the ribosomal material. Their higher specific activity after a pulse was interpreted as showing a precursor relationship to the 70S ribosomes.

Fig. 5 shows the analysis of extracts of four samples of cells taken after 10, 20, 40, and 55 minutes' exposure to C^{14} -uracil. 50S and 30S ribosomes show as shoul-

ders in the ultraviolet profile. At 10 minutes the specific radioactivities of the 50S and 30S ribosomes are at least three times that of the 70S. This difference in specific radioactivity decreases with time and has practically disappeared by 55 minutes. This long time uracil-labeling experiment appears to demonstrate qualitatively a precursor-product relationship between the native 50 and 30S and the 70S ribosomes.

When a series of cell extracts were prepared after much shorter exposures to C^{14} -

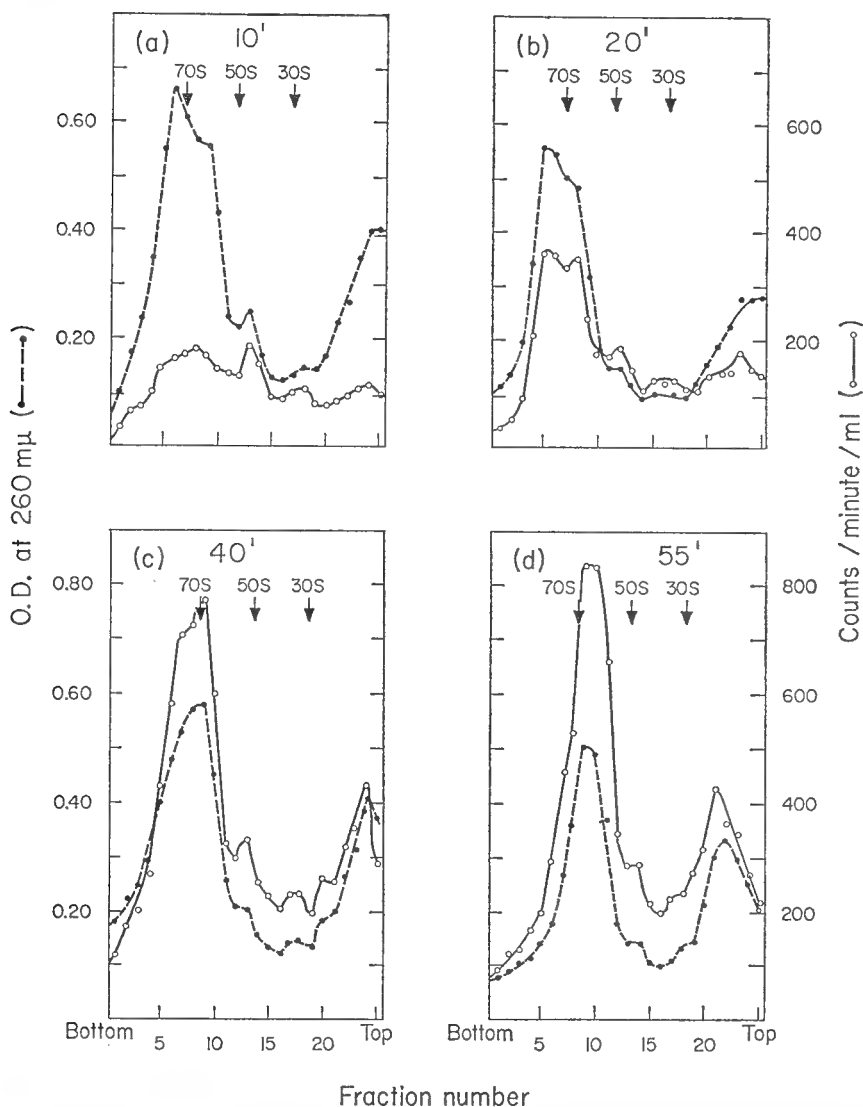


FIGURE 5 Sedimentation analysis of four total cell extracts taken from cells given (a) 10 minute, (b) 20 minute, (c) 40 minute, (d) 55 minute exposures to C^{14} -uracil. Centrifugation 90 minutes at 37,000 RPM. Cell washing, breakage, and centrifugation carried out in tris-HCl 0.01 M, pH 7.4, $MgCl_2$ 0.01 M.

uracil and analyzed in the same way (Fig. 6) the results were not interpretable on such a simple hypothesis. In this series of analyses the 50S and 30S ribosomes have a high specific radioactivity compared with that of the 70S although the ratio of specific radioactivities does not change greatly with time. In fact a simple precursor-product relationship would predict that the ratio be very large at early times if it is of the order of 3.0 at 12 minutes, as Figs. 5(a) and 6(f) show. This is evidently not the case and it appears that another process is dominant at early times causing radioactivity to appear in the 70S. This suggested direct entry of RNA into 70S

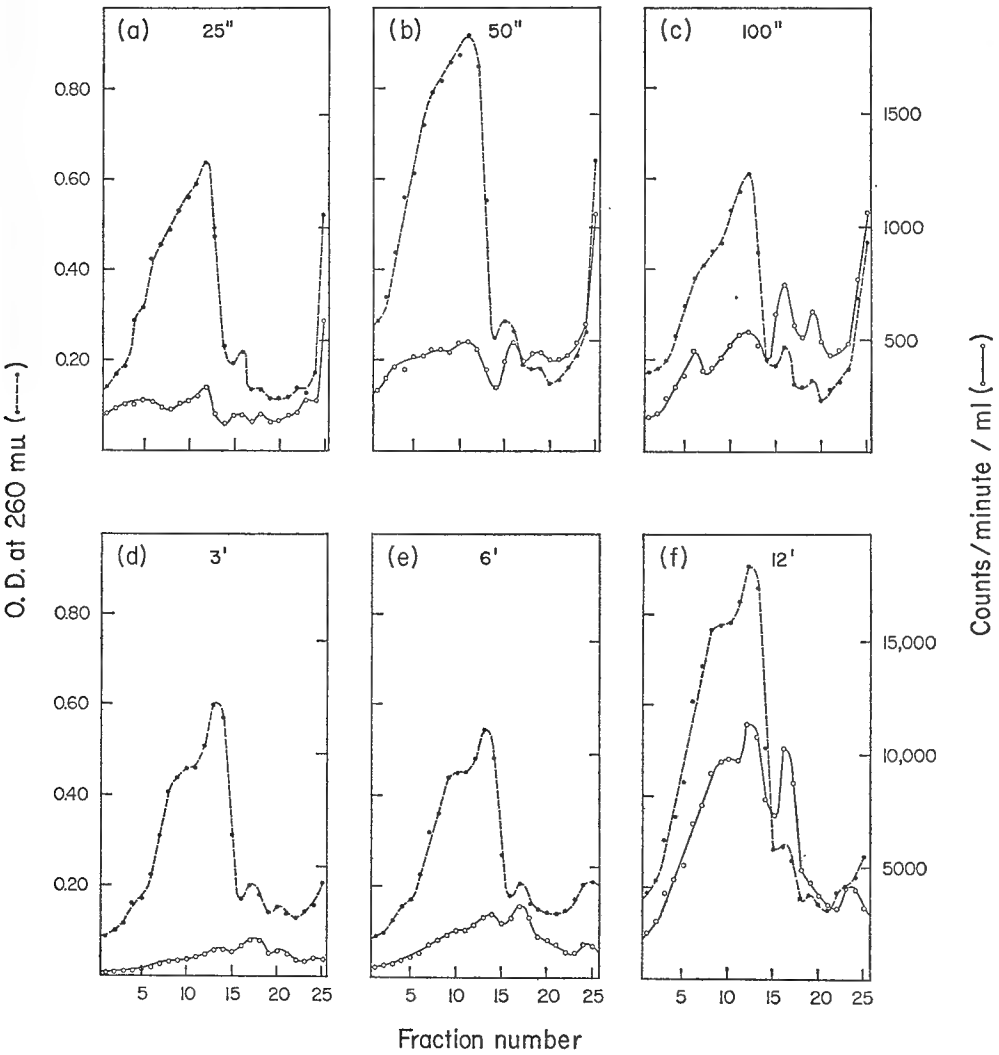


FIGURE 6 Sedimentation analysis of six total extracts taken from cells given (a) 25 second, (b) 50 second, (c) 100 second, (d) 3 minute, (e) 6 minute, (f) 12 minute exposures to C^{14} -uracil. Centrifugation 90 minutes at 37,000 RPM, 4°C. Cell washing and breakage centrifugation carried out in tris-HCl 0.01 M, pH 7.4, $MgCl_2$ 0.01 M.

without the delay brought about by passing through the large pools of 50S and 30S precursors. Another possibility was that this radioactivity was associated with the 70S peak rather than being incorporated into the large ribosomes. The increasing specific radioactivity towards the front edge of the 70S peak in Figs. 6(a) and (b) could be a result of the larger size of newly made particles or an increased quantity of radioactive material making some particles sediment more rapidly.

The next experiment was directed at selecting out the 70S ribosomes from a pulse-labeled extract and breaking them down to their constituent 50S and 30S

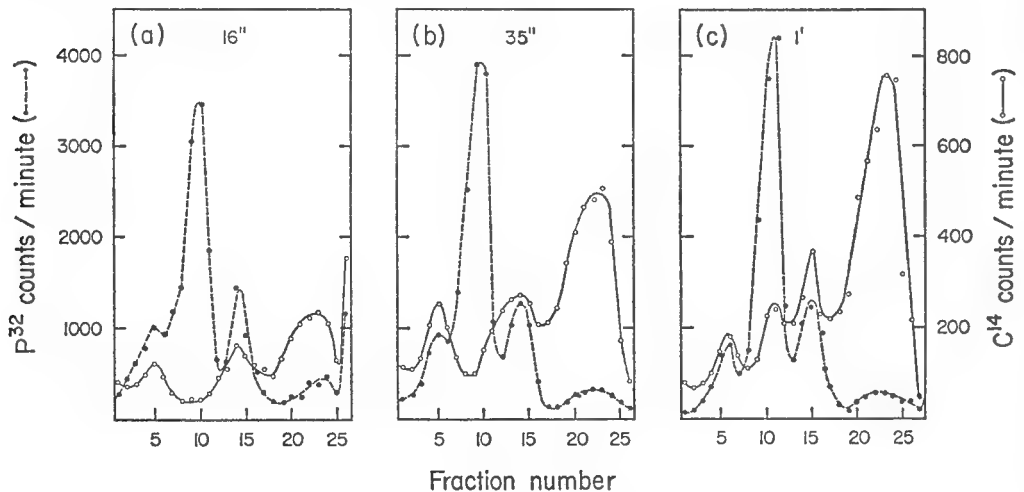


FIGURE 7 Sedimentation analysis of three 70S ribosome pellets purified by three successive 40K 30 minute centrifugations followed by a four hour dialysis against tris-HCl pH 7.4, 10^{-4} M $MgCl_2$. Cells pregrown for three generations in the presence of $P^{32}O_4^{3-}$; thus the P^{32} radioactivity (dashed line) measures the total RNA of the ribosomes. C^{14} -uracil supplied for 16 seconds (a), 35 seconds (b), and 1 minute (c). Centrifugation 95 minutes at 37,000 RPM at $4^\circ C$ in tris-HCl 0.01 M, pH 7.4, 10^{-4} M $MgCl_2$.

moieties so as to determine the location of this early radioactivity. Three samples of P^{32} steady-state labeled cells were taken after 16 seconds, 35 seconds, and one minute exposures to C^{14} -uracil, and extracts were prepared in tris HCl 0.01 M, pH 7.6, $MgCl_2$ 0.01 M. The cell walls and unbroken cells were removed by means of a five minute centrifugation at 40,000 RPM. The 70S ribosomes were selected from the supernatant by means of a thirty minute spin at 40,000 RPM and purified by two resuspensions and similar centrifugations. The final pellet, consisting largely of 70S ribosomes, contained 40 to 50 per cent of the total uracil radioactivity incorporated into RNA. The three pellets were resuspended and dialyzed against a large volume of tris HCl 0.01 M buffer containing $MgCl_2$ at 10^{-4} M for four hours. Sedimentation analysis on a sucrose gradient in 10^{-4} M magnesium produced the profiles of P^{32} and C^{14} radioactivity shown in Fig. 7. At least 90 per cent of the 70S

ribosomes have broken down into subunits of sedimentation coefficient approximately 50S and 30S. The customary 2/1 ratio in quantity of the subunits is not observed.

The distribution of C^{14} radioactivity shows that most of the pulse-labeled RNA now appears in a peak of low S number. At 16 seconds 50S ribosomes are virtually unlabeled compared with 30S ribosomes although about 20 per cent of the C^{14} remains associated with a peak of what are apparently residual 70S ribosomes. While the C^{14} radioactivity in the 30S ribosomes and in the 14S peak increase with time in Fig. 7(b) and (c), the small 70S peak becomes saturated very early with an apparent time constant of about 20 seconds. The quantity of uracil radioactivity in this component is about equal to that incorporated into total RNA during 2 seconds under the conditions of this experiment. Gros *et al.* (1961) have also observed this special component and estimate that it contains about 10 per cent of a 20 second pulse label. Thus it appears that the early label associated with the 70S ribosomes is of at least two types. The majority does not remain with the 50S and 30S after dissociation but appears in a peak of about 14S. Another rapidly saturating component remains associated with or is part of the few remaining large ribosomes having a sedimentation constant of about 70S.

Because of the complexity of analysis of cell extracts made in 10^{-2} M Mg^{++} , a search was made for conditions more suitable for the resolution of precursors from products in ribosome synthesis. Samples of cells were prepared having a steady-state P^{32} label and a 2 minute uracil pulse label. They were washed once in tris buffer containing 10^{-2} M Mg^{++} and then divided into four parts. The four samples were washed three more times in tris buffer containing magnesium chloride at 10^{-2} M, 3×10^{-3} M, 10^{-3} M, and 10^{-4} M, respectively, and finally resuspended and broken in the usual manner. All were analyzed on a sucrose gradient containing the appropriate magnesium concentration and the results are shown in Fig. 8. The first three samples were centrifuged at 37,000 RPM for 90 minutes and the last one for 150 minutes.

At 10^{-2} M Mg^{++} (Fig. 8(a)) most of the ribosomes are present as 70S but most of the radioactivity runs as 50S and 30S. The apparent specific radioactivity of the small ribosomes is greater than three times that of the 70S. Another feature worth noting is the higher specific radioactivity encountered on the leading edge of the 70S peak. Cells broken in the presence of 3×10^{-3} M Mg^{++} (Fig. 8(b)) have most of their ribosomes as 50S and 30S. At the same time about half of the radioactivity associated with ribosomes in Fig. 8(a) now appears in a peak of about 14S. There is now little difference between the specific radioactivities of the three groups of ribosomes although there is a peak of radioactivity running between the main 50S and 30S peaks. In 10^{-3} M magnesium (Fig. 8(c)), an even higher proportion of the radioactivity appears in a 14S peak, and the faster of the other two peaks of radioactivity appears to lag behind the 50S ribosome peak. The distribution of

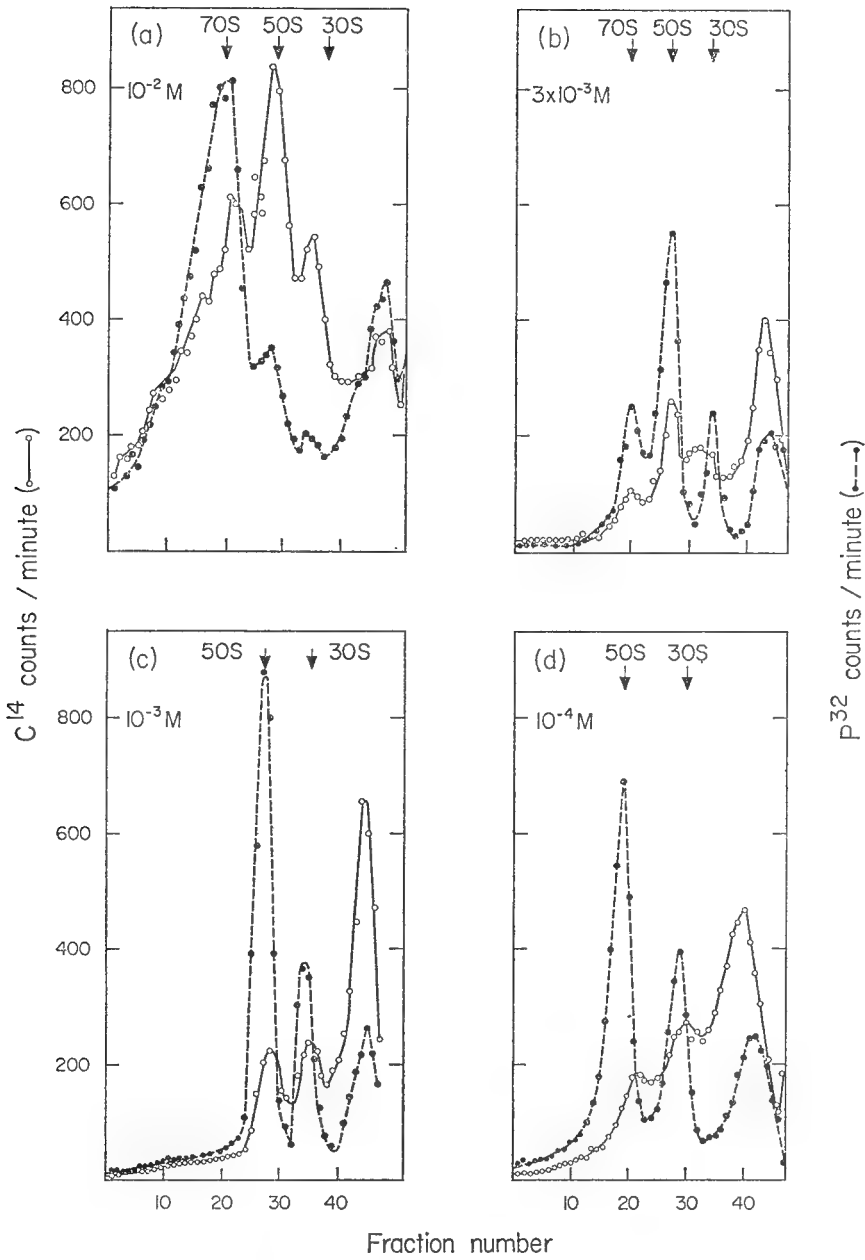


FIGURE 8 Sedimentation analysis of a total extract from cells labeled with P^{32} for four generations and with C^{14} -uracil for 2 minutes. Cells washed and extracts prepared and run in tris-HCl 0.01 M pH 7.4 containing $MgCl_2$ at (a) 10^{-2} M, (b) 3×10^{-3} M, (c) 10^{-3} M, (d) 10^{-4} M. Centrifugation (a), (b), (c) 90 minutes at 37,000 RPM, $4^\circ C$, (d) 150 minutes at 37,000 RPM, $4^\circ C$. The numerical scale refers to both P^{32} and C^{14} counts/minute. Samples analyzed correspond to 0.5 mg dry weight of cells.

radioactivity between the three objects is very similar at 10^{-4} M Mg^{++} but the increased centrifugation time in Fig. 8(d) produces higher resolution. In this instance the front-running peak of radioactivity is clearly resolved from 50S ribosomes and appears to have a sedimentation coefficient of about 43S.

Analysis of pulse-labeled, phenol-extracted RNA has already been reported in some detail (McCarthy and Aronson, 1961). All those experiments were concerned with only that fraction of RNA which was collected with the ribosome during a 4 hour centrifugation. In view of the existence of the eosome, some of which may have been lost by this procedure, further analyses of RNA were made. To obtain a total RNA fraction the cell extract was simply treated with 0.5 per cent duponol (see Materials and Methods).

In order to compare the radioactivity of the ribonucleoprotein precursors with that of the RNA, a sample of the identical cell extract that was utilized for the study of the effect of magnesium concentration (Fig. 8) was treated in this way. The results of the sedimentation analysis on a sucrose gradient (containing duponol) are shown in Fig. 9.

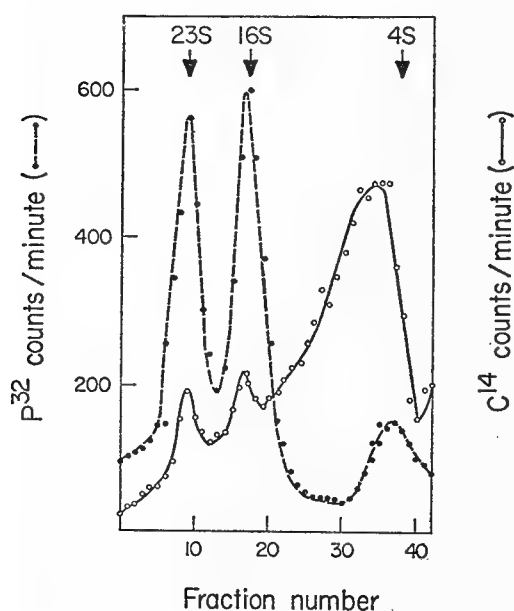


FIGURE 9 Sedimentation analysis of total RNA extracted from cells labeled with P^{32} for 4 generations and with C^{14} -uracil for 2 minutes. 0.5 per cent duponol was added to the total cell extract and then run in a sucrose gradient containing 0.5 per cent duponol in tris-HCl 0.01 M pH 7.4. Centrifugation 270 minutes at 37,000 RPM $4^{\circ}C$. The numerical scale refers to both P^{32} and C^{14} counts/minute.

Comparison of Fig. 9 with Fig. 8(d) indicates that approximately the same fraction of the RNA labeled for 2 minutes with uracil appears as complete 16S and 23S molecules, as that associated with the 30S and 43S peaks in the total extract. The rest of the C^{14} label appears in a peak of approximately 8S. Evidently the eosome appearing as 14S in the total extract appears as only 8S when treated with either duponol or phenol (McCarthy and Aronson, 1961; Gros *et al.*, 1961).

E. RESOLUTION OF THE RIBOSOME PRECURSORS BY SEDIMENTATION

The resolution of early labeled RNA from the bulk ribosomes suggested that these conditions of cell breakage and analysis of extracts would be suitable for the determination of the details of the kinetics of ribosome synthesis. At 2 minutes two objects other than the 50S and 30S ribosome peaks were highly labeled and readily resolvable. Further experiments were carried out to study their possible roles as ribosome precursors.

For this purpose careful analysis was made in 10^{-4} M magnesium of six samples of extracts prepared from P^{32} steady-state-labeled cells given from 30 seconds to 12

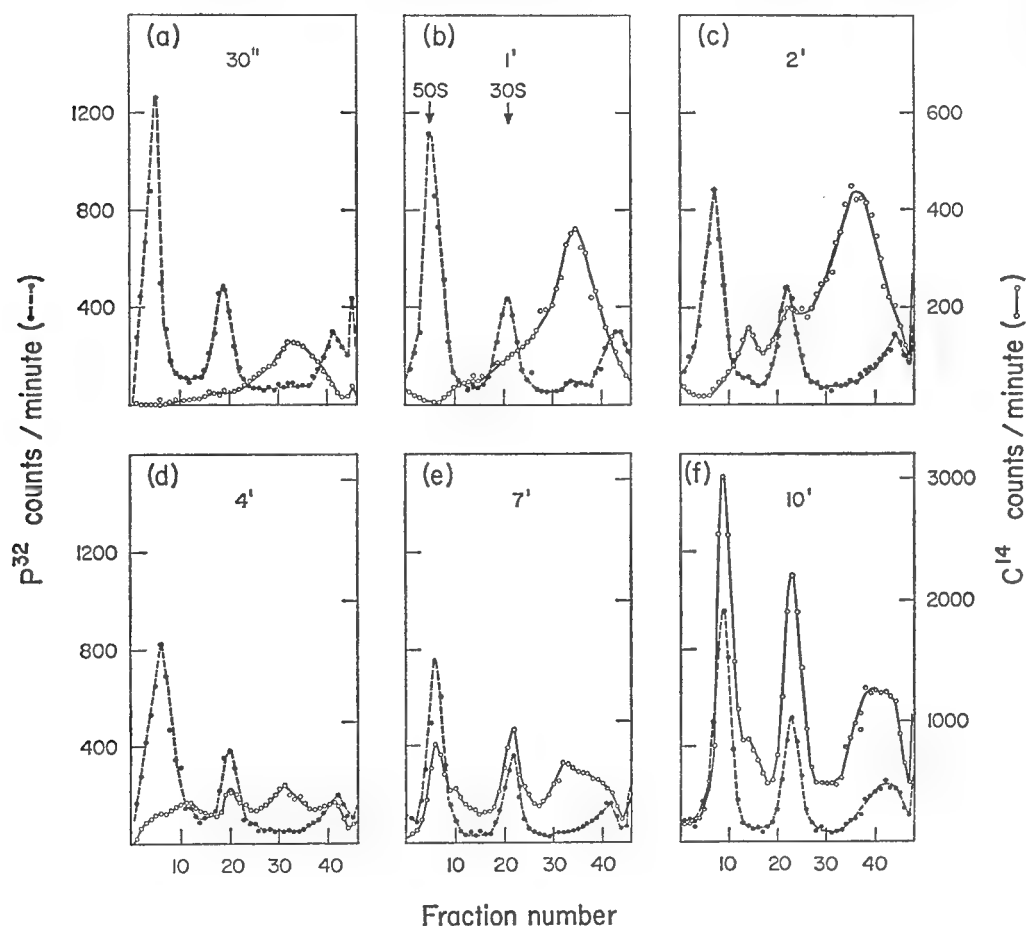


FIGURE 10 Sedimentation analysis of six DNAase-treated total extracts made and centrifuged in tris-HCl 0.01 M, pH 7.4 $MgCl_2$ 10^{-4} M from cells steady-state-labeled with P^{32} given (a) 30 second exposure to C^{14} -uracil, (b) 1 minute, (c) 2 minute, (d) 4 minute, (e) 7 minute, (f) 10 minute. Centrifugation 175 minutes at 37,000 RPM $4^\circ C$. Samples analyzed correspond to 0.5 mg dry weight of cells.

minutes exposure to C^{14} -uracil (Fig. 10). The extracts were treated with DNAase prior to centrifugation through a sucrose gradient. The P^{32} profile shows three main peaks of 50S and 30S ribosomes and soluble RNA. In Figs. 10(a) and (b) the C^{14} radioactivity appears almost entirely in the rather broad peak at about 14S. After 2 minutes there is little apparent rise in the specific radioactivity (C^{14}/P^{32} ratio) of this peak.

At 2 minutes two other peaks are clearly distinguishable; one associated with 30S ribosomes and another appearing between 30S and 50S at approximately 43S. The 43S component continues to rise in figures 10(d) and (e). Up to 7 minutes there is very little C^{14} radioactivity which can be associated with the 50S peak but then the radioactivity increases by a factor of about 10 between 4 minutes and 10 minutes.

A preliminary interpretation of this data would suggest that all the radioactivity passing into ribosomal RNA is delayed by about a 2 minute pool of material having a mean sedimentation coefficient of about 14S. By analogy with the column analysis this first precursor would be the eosome. A second intermediate stage can be distinguished in which C^{14} -uracil radioactivity appears in the region of 43S associated with only a small fraction of the total ribosomal material. This appears to be a part of the neosome or second stage fractionated by column analysis. A quantitative examination in the manner previously described (Paper II), of the time course of labeling of the various peaks was therefore undertaken in order to clarify this initial impression.

Measurement of the C^{14} -uracil radioactivity present in the various components is considerably easier in the case of the sedimentation analyses than in the DEAE column analyses already described. The resolution between the various objects is more complete and the peak shapes are clearly defined by the P^{32} radioactivity.

Kinetic analysis was carried out in a series of steps analogous to those already described for the DEAE column analysis.

(a) The sample times were converted to τ and the ratio of the total C^{14} radioactivity to the total P^{32} radioactivity (corrected for decay) was obtained for each time. ϕ_T was then evaluated by the procedure already described.

(b) The next step was to determine the relative flows into the 30S and 50S ribosomes from the eosome pool. Since the total 30S material in the cell is one half of the 50S material, these flows would be one-third and two-thirds of the total if the two pathways were independent. ϕ_{30} was calculated for the 30S region using the peak shape defined by the P^{32} count. All C^{14} counts associated with regions of S number higher than 30 were summed to obtain ϕ_{43+50} , presuming because of its sedimentation constant that the 43S peak was a precursor of the 50S. These two functions are plotted in Fig. 11. It is immediately clear that the curves are not a factor of two apart and especially at early times the 30S region has more than its share of radioactivity. It appears that a precursor of the 50S ribosome is present in the 30S region.

(c) In a more detailed analysis ϕ_E (eosome), ϕ_{43} , and ϕ_{50} were evaluated. At early times ϕ_{30} and ϕ_{43} are not known very precisely due to contamination by the trail of eosome. The peak shape of eosome, however, is such that reasonable estimates can be made. Further, ϕ_E cannot be estimated directly at late times but must be corrected for the growing radioactivity of S-RNA. In making this correction the quantity of S-RNA was evaluated from the P^{32} radioactivity in the region of small sedimentation coefficient. Its specific radioactivity was taken to be equal to that of the total RNA (ϕ_T), since there is no evidence for a delay in its labeling. In the case of the 43S reasonably precise estimates can be made out to 10 minutes due to the strong depression of ϕ_{50} and an accurate knowledge of the peak shape obtained from Fig. 10(c).

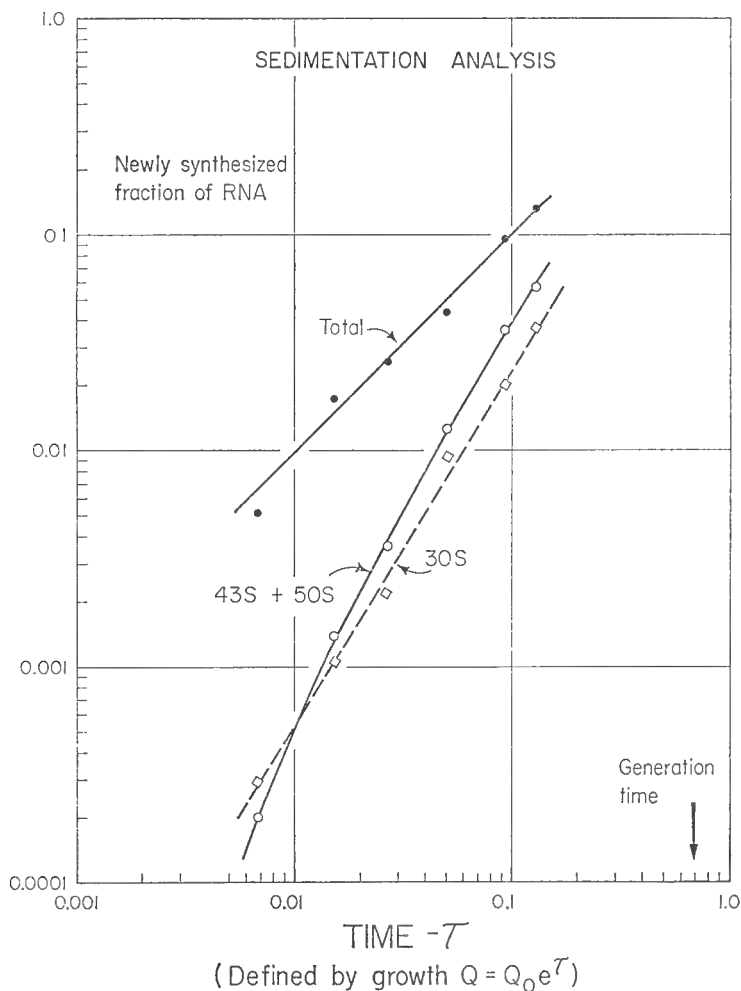


FIGURE 11 Log-log plot of the newly synthesized fraction of RNA present in total cell extract (●), 30S region (□), and 43S + 50S region (○) as a function of time. Data from Fig. 10.

(d) The three functions ϕ_E , ϕ_{43} , and ϕ_{50} are plotted together with ϕ_T in Fig. 12. The curve for ϕ_E is essentially the same as that obtained by column analysis (Fig. 4). The value at which it levels off indicates a pool size of about 2 per cent of the total ribosomal RNA for the eosome. Since the eosome accounts for the total flow at early times both ϕ_{43} and ϕ_{50} are strongly depressed at early times. ϕ_{43} is proportional to τ^2 at early times and ϕ_{50} to τ^3 . This would be expected if the 43S component is the neosome or second stage in the synthesis of the 50S ribosome.

The objects first to receive radioactivity, variously called 14S RNA, eosome, informational RNA, or messenger RNA are probably of great importance to the

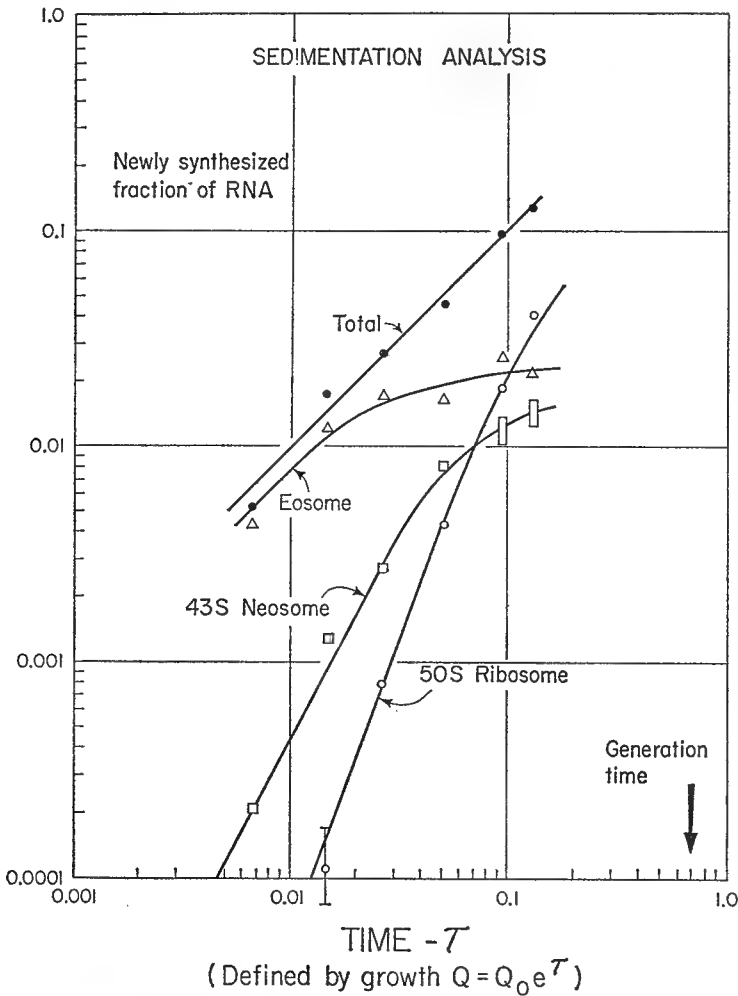


FIGURE 12 Log-log plot of the newly synthesized fraction of RNA present in the total cell extract (●), eosome (Δ), 43S neosome (□), and 50S ribosome (○) as a function of time. Data from Fig. 10. The curve drawn for the 50S ribosomes is the calculated curve shown on Fig. 3 multiplied by two-thirds since the 50S ribosomes account for two-thirds of the ribosomal nucleoprotein. The agreement is striking.

central problem of the origin and function of ribosomal RNA. Therefore the evidence showing that the RNA in the 14S region is precursor to ribosomal RNA will be examined in detail. For this purpose the more conventional linear plot of the time course of labeling, shown in Fig. 13 is a useful adjunct to the curves shown in Figs. 11 and 12.

On Fig. 13 are shown first the radioactivity in the total RNA, ϕ_T' (reduced by 15 per cent to correct for the soluble RNA), second the radioactivity of the 14S region, ϕ_E , corrected at late times for the soluble RNA, as described above, and third the radioactivity of neosome plus ribosome, $\phi_{30} + \phi_{43} + \phi_{50}$ called here ϕ_R' .

All of the radioactivity initially enters the eosome, thus at early times $\phi_T' = \phi_E$. Later ϕ_E levels off at 0.02 corresponding to 2 per cent of the total ribosomal RNA.

If the total flow to ribosomal RNA passed through an object of this size the time constant would be about 2 minutes which is consistent with the shape of the curve for ϕ_E .

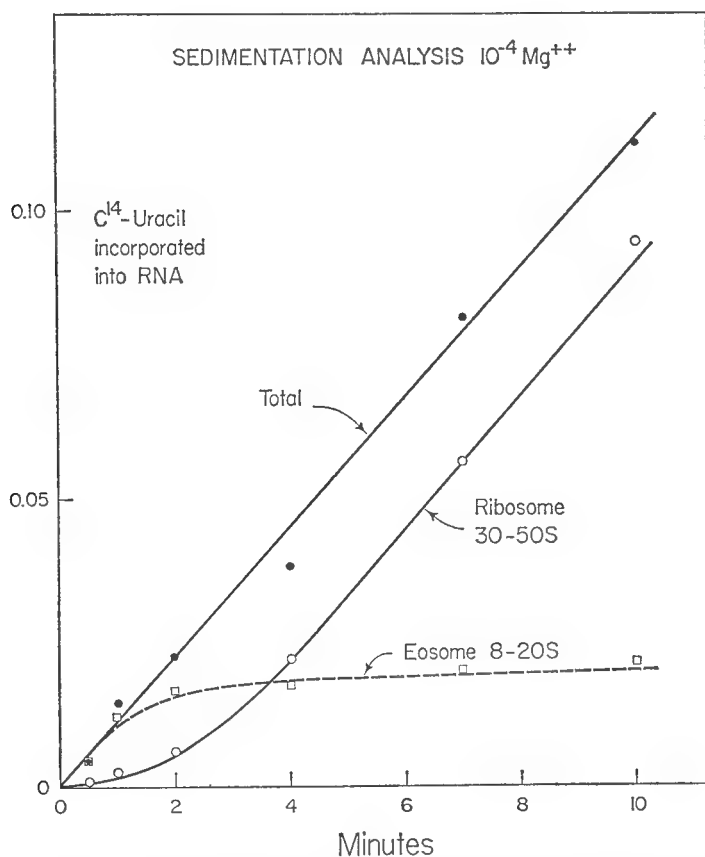


FIGURE 13 The C^{14} -uracil label incorporated into total RNA (●), the eosome region 8-20S (□), and the ribosome region (○), *i.e.*, material between 30S and 50S including neosome and ribosome. Data from Fig. 10. The label in each component is plotted as ϕ functions ϕ_T , ϕ_E , ϕ_{R+E} as in Figs. 11 and 12.

The curve for ϕ_R' has zero slope at zero time showing that the entry of radioactivity is delayed by a precursor. Further, the ribosome curve, ϕ_R' falls below ϕ_T' at late times by an amount equal to the quantity of the eosome. Thus the delay of ϕ_R' is approximately correct for the size of the eosome. While ϕ_R' rises initially in proportion to τ^2 the values at 30 seconds and 1 minute are lower than would be expected. Using the equation shown in Paper II, Fig. 1, for the earliest time points, the calculated quantity of precursor is 3.5 per cent of the total ribosomal RNA. This difference may be due to an underestimate of ϕ_{30} or may indicate that the quantity of eosome is somewhat greater than 2 per cent, as does the DEAE analysis. Alternatively, there may be a time required for completion of the eosome before it can be utilized for neosome synthesis. In any case it is clear that very little if any C^{14} -uracil radioactivity bypasses the eosome on its way to neosome or ribosome.

F. DISCUSSION

From the analysis of uracil-labeled cell extracts, it is clear that at early times all the radioactivity is present in a fraction, named eosome, peaking at 14S. The kinetic behavior of the component has been studied and its size estimated at 2 to 3 per cent of the total ribosomal RNA. It can be shown from the same experiments that label entering nucleoprotein of higher molecular weight is delayed by a component of about the same size. Together these observations suggest that the eosome is predominantly precursor to ribosomes.

In another recent study (Gros *et al.*, 1961) however, the rapidity of labeling of the eosome has been invoked as a criterion of turnover. These authors have suggested that the rapid rate with which label enters and leaves the eosome fraction proves instability of these RNA molecules. In general, however, it is clear that the rapid labeling of a small component indicates merely that the flow into it is much larger than that required to maintain its quantity in the growing cell. The loss of label when an excess of unlabeled isotope is added shows only the already obvious fact that there is also a transfer of material out of this component. These qualitative measurements cannot be expected to distinguish between a precursor through which label flows to a product and a compound which is synthesized and then is broken down to its constituent parts. To some extent, the lack of distinction between these two alternatives results from the use of the word turnover without properly stating the level of turnover that is being considered. In order to distinguish between the two very different alternatives it would be preferable if the term were reserved for phenomena of molecular instability since the word precursor is an adequate description of the other alternative.

The difficulty of demonstrating true turnover in the sense of synthesis and degradation of a given molecule has already been discussed (Paper I). What we have demonstrated is that there exists a precursor-product relationship between the eosome and the product ribosomes for the transfer of C^{14} -uracil radioactivity.

It does not prove conclusively that labeled molecules initially observed as eosomes are incorporated *as such* into completed ribosomes. An alternative involves breakdown of the eosome molecules and a synthesis of ribosomes by means of a quantitative reutilization of the labeled degradation products. It should be pointed out that in common with many similar tracer studies the latter alternative cannot be rigidly ruled out. In order to conserve the label for quantitative reutilization, the breakdown has to occur in such a way that there is no mixing with other unlabeled RNA precursors (Paper I). In the absence of evidence for such a breakdown of

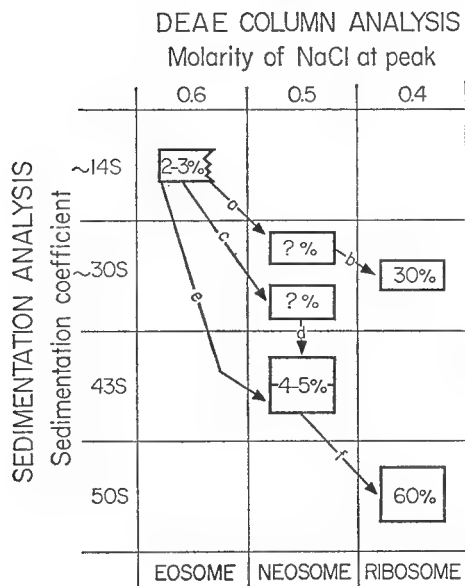


FIGURE 14 Schematic diagram of the flow of RNA in ribosomal synthesis. The diagram indicates the way in which the information derived from DEAE-cellulose column analysis and sedimentation analysis has been combined to give a more complete scheme. The figures in the boxes represent the percentages of the total ribosomal RNA existing in each of the states in a steadily growing cell. Movement downwards is a result, principally, of the completion of RNA subunits. Movement to the right is principally a result of the addition of protein.

eosome molecules, the data have been interpreted in terms of a precursor-product relationship.

As far as the details of ribosome synthesis are concerned it is clear that three main stages may be described. The clearest demonstration derives from the rate of labeling of the main ribonucleoprotein peak in the column analysis. Its radioactivity rises initially as the cube of time. The total label present at early times is so low as to require two sequential precursors. The total precursor material can be only 10 per cent of the total RNA and a single precursor of this magnitude could not introduce such a delay into the product (Paper II, Fig. 1).

Separation of the total precursor material into the two components is, in fact, achieved by the DEAE-cellulose chromatography. The primary eosome stage can be shown to be 2 to 3 per cent of the total ribosomal RNA and the second neosome stage 6 to 7 per cent. The time variation of the label entering the second peak shows it to be delayed by one precursor.

Sequential precursors are also clearly shown by sedimentation analysis. The 50S

peak is very well resolved from precursor material and shows the same time course of labeling as the main ribonucleoprotein peak in column analysis. As shown by a comparison of Fig. 3 and Fig. 12 the radioactivity of the 50S is just two-thirds of the main ribonucleoprotein peak as would be expected since the 50S ribosome makes up two-thirds of the 70S ribosome.

The diagram shown in Fig. 14 summarizes the results obtained with both types of analysis and indicates the correlation between the two. In the following paragraphs the evidence which supports each of the features of this diagram will be considered.

Eosome. The interpretation of the role of the eosome as precursor to all of the ribosomal RNA has been discussed above. It is not to be supposed that the eosome is one homogeneous class. It has a broad range of sedimentation constants and presumably supplies the RNA to a variety of ribosomes. In particular the nucleotide compositions of the 30S and 50S ribosomes differ (Bolton, 1959). The peak at 0.6 M in the column analysis and the broad region peaking at 14S in the sedimentation analysis both carry all of the radioactivity at early times and later saturate at about the same quantity of radioactivity.

Flows a and b and the 30S Neosome Precursor to 30S Ribosomes. It is clear from the column analysis that there exists a neosome precursor to 30S ribosomes since the peak eluted at 0.4 M contains both the 30S and 50S ribosomes, the radioactivity entering it is very strongly delayed and rises as τ^3 at early times. The only location in the sedimentation analysis (Fig. 10) where such an object can occur is in the 30S region. Because of the lack of resolution it has been impossible independently to assess its kinetics of labeling or measure its quantity. After correction for the trails of 43S and eosome peaks in Figs. 10(c) and (d), it appears that the C^{14} radioactivity in the 30S region reaches its maximum somewhat behind the P^{32} peak corresponding to the 30S ribosomes. This result is quite uncertain but leaves an impression of heterogeneity in the 30S region.

Flows c and d and the 30S Precursor to 50S Ribosomes. Fig. 11 shows that there is a greater flow into the 30S region than is required for the synthesis of the 30S ribosomes, in fact more than half the flow from the eosome passes into the 30S region at early times. This shows that a part of the flow that ultimately reaches the 50S ribosome passes through a 30S neosome. We have chosen to indicate on the diagram that one half of the flow to 50S passes this way for the following reasons. In the first place the 43S radioactivity (Fig. 12) is one-third of the neosome radioactivity (Fig. 4) at early times. In the second place a sedimentation coefficient of 30S suggests that one-half of the 50S RNA is already present. It is not clear whether the two 30S neosomes indicated are identical to each other. The nucleotide compositions of the 50S and 30S ribosomes differ but this difference could be made up through the flow e. The total quantity of the 30S neosomes can be crudely estimated by the difference between the amount of 43S neosome (4 to 5 per cent) and the total amount of neosome (7 per cent) indicated on Fig. 4. No estimate of

the relative quantities of the two objects indicated can be made if in fact they differ.

The 43S Neosome and Flows e and f. The 43S neosome stands out clearly in sedimentation analysis (Fig. 10(c)) at the appropriate time. The time course of labeling shown on Fig. 12 is that of the neosome or second stage in the sequence. The radioactivity rises as τ^2 initially and levels off later. At early times the curve has the shape of ϕ_N (Fig. 4) but only one-third of the magnitude of ϕ_N . If the total flow to the 50S ribosomes passed directly to the 43S from the eosome, ϕ_{43} would be expected to be just two-thirds of ϕ_N . Therefore it is clear that about half the flow to 50S ribosomes passes to the 43S from the eosome.

It appears certain that no eosomal RNA goes directly to the 50S ribosome because ϕ_{50} is proportional to τ^3 at early times. The 43S neosome is shown on the diagram as containing the full complement of RNA of the 50S ribosome. This seems likely from its sedimentation constant.

The quantity of 43S neosomal RNA can be estimated to be about 5 per cent from the steady-state P^{32} radioactivity that remains in this region after the 30S and 50S contributions have been subtracted, assuming reasonable and symmetrical peak shapes. The specific radioactivity estimated on this basis at early times is just one-half what would be expected if the total flow to 50S ribosomes passed directly from the neosome to the 43S. This is, of course, consistent with the diagram since one-half of the flow should be delayed by the 30S neosome precursor to the 43S.

The diagram (Fig. 14) shows a set of sequential relationships which are more complex than the two sequential precursors shown on Fig. 3. The diagram suggests that half of the radioactivity of the 50S ribosomes should rise in proportion to τ^4 for a time while the other half should rise as τ^3 . All of the 30S ribosome radioactivity should rise as τ^3 .

While deviations from the 3-stage model showed clearly in the time course of labeling precursors such as the 30S and 43S neosomes, the accuracy of the data is not adequate to resolve the predicted fourth power component in the final product.

These studies of the synthesis of the RNA portion of ribosomes provide only a few hints to the concurrent process of the addition of protein. The step from 43S neosome to 50S ribosome involves both a change in sedimentation coefficient and a change in the elution from DEAE, but no additional RNA is added. It seems quite obvious that this change is due to a change in the protein content. Studies using C^{14} -leucine to follow the synthesis of the protein are described in the following communication (Paper IV).

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Comment. Studies of the composition of nascent RNA (III.B.5) and analysis using DNA agar columns (III.B.6) show that the eosome fraction contains roughly 33 per cent DNA-like RNA. Richard B. Roberts.

III.B.3 The Synthesis of Ribosomes in *E. coli*, 4, The Synthesis of Ribosomal Protein and the Assembly of Ribosomes

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ABSTRACT The incorporation of C^{14} leucine into the protein moiety of ribosomes has been studied as a sequel to the studies of ribosomal RNA synthesis. In contrast to the latter studies, labeled leucine is incorporated directly into 50S and 30S ribosomes without measurable delay by precursor stages. There is, however, evidence of some transfer of radioactivity from the 43S group of particles to the 50S. The inhibition of protein synthesis by chloramphenicol results in the accumulation of material similar to the eosome—the primary precursor in ribosome synthesis. There is also evidence for the synthesis of some neosome. The results of the studies of ribosomal RNA and protein synthesis are combined into a model of ribosome synthesis. Finally, consideration is made of the significance of these studies of ribosome synthesis for general problems of protein synthesis and information transfer.

A. INTRODUCTION

The previous paper reports studies of the synthesis of ribosomal RNA. These studies indicated several states of organization of ribosomal RNA during the assembly of the complete 50S and 30S ribosomes. The two precursor stages of ribosome synthesis apparently differed from complete ribosomes in having a considerably lower content of protein. Study of the synthesis of ribosomal protein was therefore undertaken to elucidate the details of the process of assembly of RNA and protein to make completed ribosomes.

Results are presented showing the kinetics of incorporation of leucine into ribosomal protein. The radioactivity due to structural ribosomal protein has been separated from that of nascent protein synthesized on the ribosomes (McQuillen, Roberts, and Britten, 1959) by means of chase experiments. Finally, the general features of ribosome synthesis are summarized together with a discussion of the role of ribosomal RNA and other RNA fractions in protein synthesis.

B. MATERIALS AND METHODS

Methods of growing cells, estimating radioactivity, and fractionating cell extracts have already been described in Paper III.

The C^{14} -leucine used as a protein label was purchased from the New England Nuclear Corporation and had a specific radioactivity of 5.2 mc/mm. A P^{32} steady-state label was used as a measure of total RNA as before.

RNA synthesized during inhibition with chloramphenicol was studied with the aid of 2- C^{14} -uracil as previously described.

C. RESULTS

1. *Analysis of Pulse Labeled Extracts by Sedimentation.* Most previous sedimentation analyses have been made in higher concentrations of magnesium (10^{-2} M) in which 70S and larger ribosomes survive (Roberts, 1960). The results were only qualitative but led to the conclusion that the 50S and 30S ribosomes present in the cell extract were the precursors of the 70S. Since the present experiments were designed to complement those in which C^{14} -labeled uracil was used, the techniques and methods of analysis were completely analogous, including the use of a steady-state P^{32} label as a measure of total ribosomes and the breakage of the cells in 10^{-4} M magnesium.

Fig. 1 shows the sedimentation analysis of five total cell extracts prepared from cells given exposures to C^{14} -leucine varying from 30 seconds to 8 minutes. The P^{32} profile shows the usual three major peaks of 50S ribosomes, 30S ribosomes, and DNA and S-RNA. The C^{14} radioactivity associated with the 50S and 30S peaks is already high at 30 seconds suggesting that much of it represents nascent protein (McQuillen, Roberts, and Britten, 1959) rather than ribosomal protein. At 30 seconds about one-third of the leucine radioactivity is associated with ribosomes, indicating that the amount of nascent protein found in association is equivalent to some 10 seconds' total supply, *i.e.* 0.2 per cent. This figure is higher than that previously reported by McQuillen *et al.* This could be a result of the different method of breakage preserving more of the protein-ribosome association.

At later times the radioactivity of the nascent protein is less prominent and there is a continued rise in the amount of C^{14} radioactivity in the 50S and 30S ribosomes. In addition, the peak of high specific radioactivity (C^{14}/P^{32} ratio) between the main 50S and 30S ribosomes visible in Fig. 1(b) continues to increase. This radioactivity may be attributed to an object of about 40-45S presumably the same as the 43S neosome previously described (Paper III).

As far as the entry of C^{14} radioactivity into the 50S and 30S ribosomes is concerned there is little sign of any delays brought about by a precursor through which all the ribosomal protein must pass. The rising specific radioactivity from the very earliest times shows that most of the ribosomal protein enters the 50S and 30S directly.

Since a high proportion of the C^{14} -leucine radioactivity enters 50S and 30S ribosomes directly, it is clear that short labeling periods are necessary to demonstrate any more detailed features of ribosomal protein synthesis. In view of the contribution of nascent protein to the total ribosomal protein at early times, a short pulse of C^{14} -leucine followed by a chase with C^{12} -leucine is useful to reveal the transfer of ribosomal protein. Cells were labeled with P^{32} for three generations and then with C^{14} -leucine for one minute when a 100-fold excess of C^{12} -leucine was added. Samples were taken after 10 seconds and 1 minute, 2 minutes and 20 seconds, 4

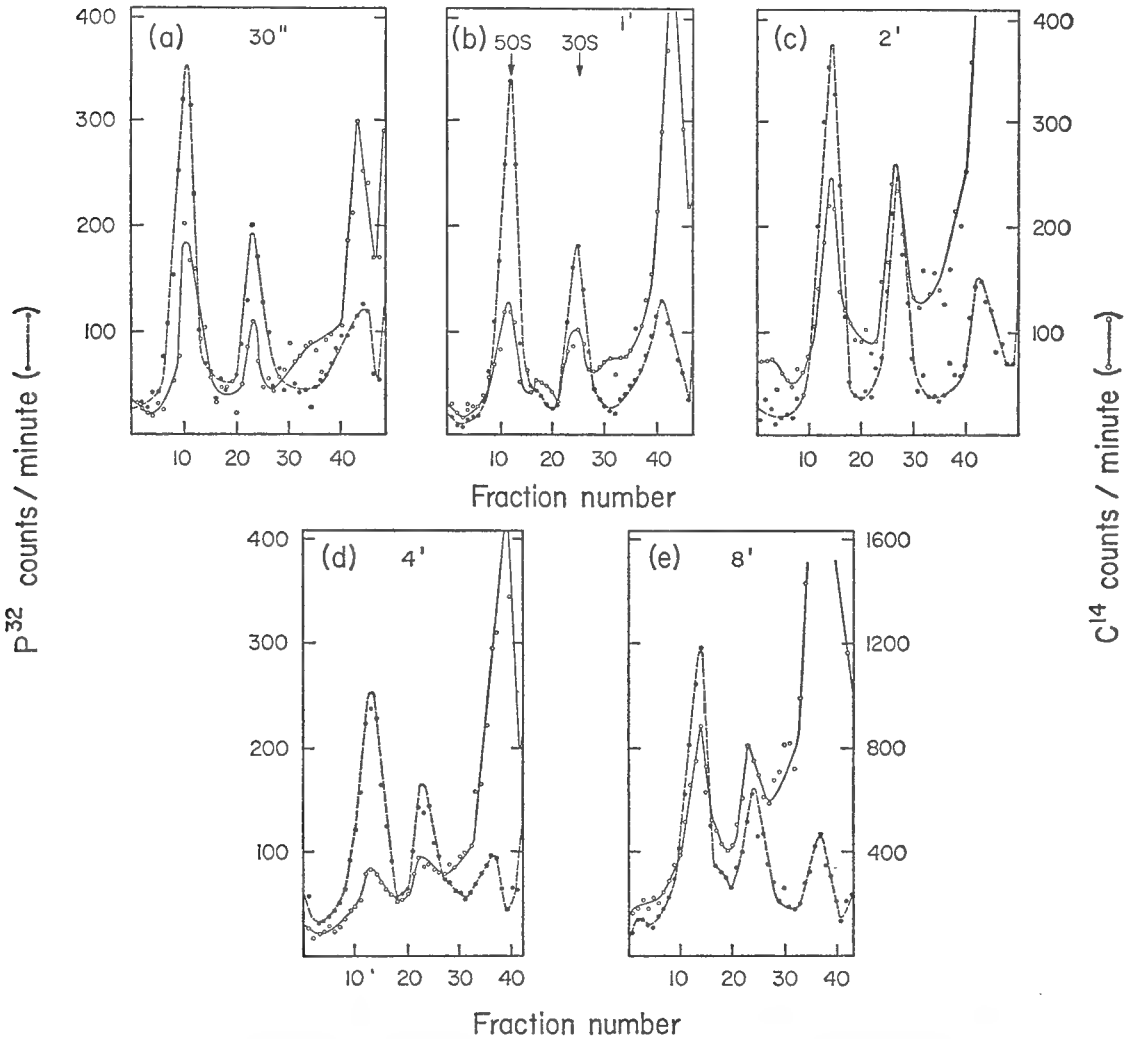


FIGURE 1 Sedimentation analysis of five total cell extracts from cells labeled with P^{32} for four generations and given (a) 30 seconds, (b) 1 minute, (c) 2 minutes, (d) 4 minutes, (e) 8 minutes exposure to C^{14} -leucine. Cells washed and extracts prepared in tris-HCl 0.01 M pH 7.4 $MgCl_2$ 10^{-4} M. Centrifugation 160 minutes at 37,000 RPM $4^{\circ}C$.

minutes, and 7 minutes and 45 seconds. Fig. 2 shows the efficiency of the chase in stopping further incorporation of radioactivity into protein.

Extracts were prepared in 10^{-4} M magnesium in the usual way and analyzed by sedimentation. Three of the analyses are shown in Fig. 3. Preliminary examination of the radioactivity profiles reveals that the 50S peak gains radioactivity relative to the 30S, and that the label in the 43S decreases as a function of time. The C^{14} present in the 30S, 43S, and 50S peaks was computed for all five analyses in the usual way

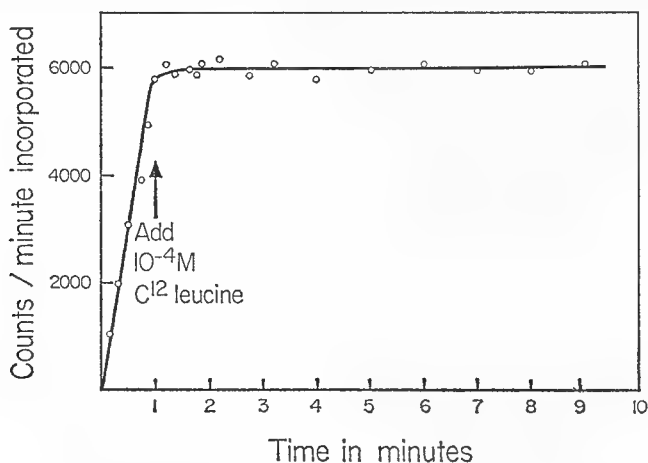


FIGURE 2 The incorporation of C^{14} -leucine (10^{-8} M) into protein in a culture of *E. coli* ML 30. C^{12} -leucine was added to a final concentration of 10^{-4} M at one minute.

(Paper III) and corrected for sample size and cell growth. These three functions are plotted in Fig. 4. It can be seen that the increase in radioactivity in the 50S is paralleled by a decrease in the 43S within limits of error. On the other hand the radioactivity of the 30S falls only by the factor expected for cell growth.

It should be pointed out that with this method of analysis by sedimentation the 30S peak always contains much more protein than one would expect. In general even with long labeling periods the quantity of protein associated with the 30S peak is almost equal to that associated with the 50S. In the present chase experiment about 35 per cent of the total protein is associated with ribosomes compared with the 25 per cent or so expected on the basis of the number of ribosomes and their protein content. This suggests that while the 50S contains the expected quantity of protein, the proportion of protein in the 30S is unexpectedly high.

2. *Effect of Chloramphenicol on Ribosome Synthesis.* The addition of chloramphenicol to a culture of *E. coli* immediately inhibits protein synthesis. On the other hand RNA and DNA synthesis continue at approximately the normal rate for the first 30 minutes or so (Gale and Folkes, 1953). During long periods of inhibition a special fraction of RNA can accumulate to a high level (Pardee, Paigen, and Prestidge, 1957) and preexisting ribosomes may be degraded (Nomura and Wat-

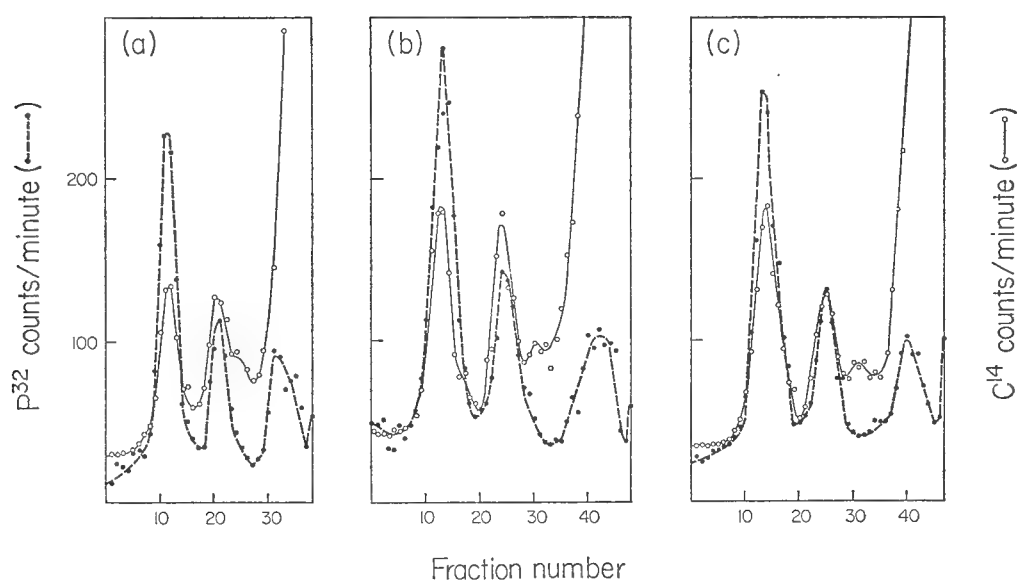


FIGURE 3 Sedimentation analysis of three total cell extracts prepared from cells labeled with P^{32} for four generations and C^{14} -leucine for one minute followed by (a) 10 seconds, (b) 2 minutes and 45 seconds, (c) 7 minutes and 45 seconds in C^{12} -leucine (See Fig. 2). Cells washed and extracts prepared in tris-HCl 0.01 M pH 7.4 containing $MgCl_2$ 10^{-4} M. Centrifugation 165 minutes at 37,000 RPM $4^\circ C$. The numerical scale refers to both P^{32} and C^{14} counts/minute.

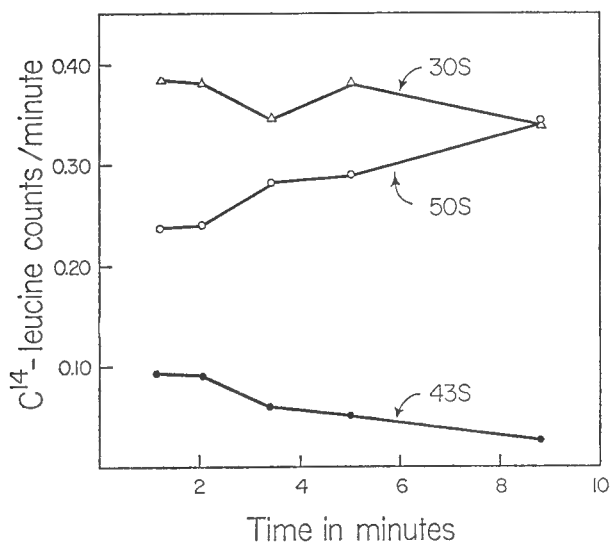


FIGURE 4 The C^{14} -leucine radioactivity in the 30S, 43S and 50S components as a function of time after a one minute pulse and chase. Data from Fig. 3.

son, 1959). Finally, removal of chloramphenicol results in the degradation and loss of much of the chloramphenicol RNA (Neidhart and Gros, 1957).

The overall base composition of RNA synthesized in the presence of chloramphenicol is identical to normal bacterial RNA (Pardee and Prestidge, 1956). Fractionation of the RNA produced on a column of DEAE cellulose gives two components, one having the base composition of soluble RNA and the other that of ribosomal RNA (Bolton, 1959). It was therefore of considerable interest to examine the relationship of the RNA formed to the normal stages of ribosome synthesis.

With the experience already gained in studies of the incorporation of uracil into ribosomes (Paper III) a period of labeling in the presence of chloramphenicol was chosen such that in the control culture most of the label would be present as product ribosomes and yet the eosome and 43S neosome components would still be visible. A culture of *E. coli* ML 30 was randomly labeled with P^{32} for three generations and then split into three equal fractions. To two of these chloramphenicol was added at concentrations of 50 $\mu\text{g}/\text{ml}$ and 200 $\mu\text{g}/\text{ml}$. After two minutes an equal quantity of C^{14} -uracil was added to each culture at 10^{-5} M. After 10 minutes each culture was harvested, washed, and extracts prepared in tris-HCl 0.01 M, pH 7.4 containing DNAase at 1 $\mu\text{g}/\text{ml}$. The two chloramphenicol-inhibited cultures incorporated 80 per cent as much C^{14} -uracil as did the control.

The sedimentation analysis of two of the cell extracts is shown in Fig. 5. As the

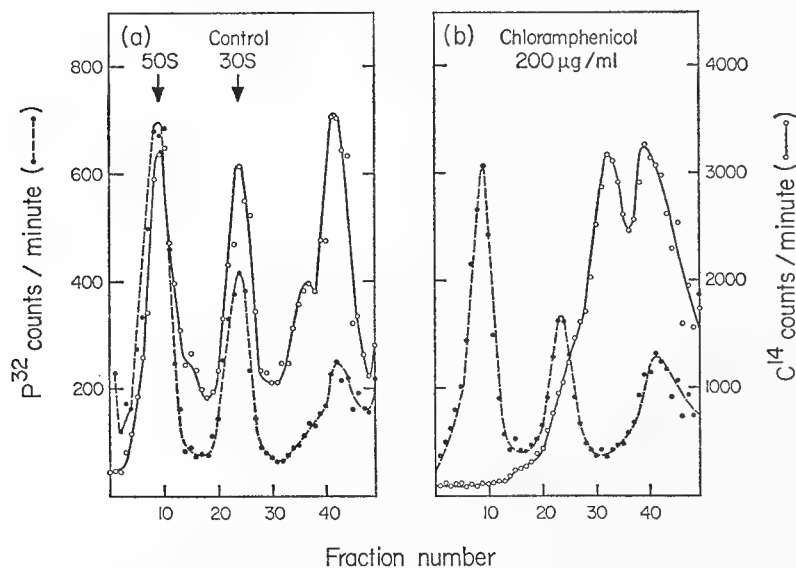


FIGURE 5 The effect of chloramphenicol on the incorporation of C^{14} -uracil into ribosomes. Cells were grown for three generations in P^{32} and given a 10 minute exposure to C^{14} -uracil. (a) control, (b) in the presence of chloramphenicol 200 $\mu\text{g}/\text{ml}$ added two minutes before the uracil. Cells washed and extracts prepared in tris-HCl 0.01 M pH 7.4 MgCl_2 10^{-4} M in the presence of DNAase. Centrifugation 160 minutes at 37,000 RPM 4°C .

analysis of the two chloramphenicol-inhibited cultures were identical, only one is shown. In the control culture most of the C^{14} -uracil is present in the 50S and 30S ribosome peaks and S-RNA. However shoulders of neosome at 43S and eosome at 14S are still visible. In the extract from chloramphenicol-inhibited cells most of the labeled uracil is associated with material of sedimentation coefficient less than 20. Moreover there is a separation into two peaks, one coincident with S-RNA and the other at the position occupied by eosome precursor in short pulse experiments (Paper III).

There are, however, signs of other minor labeled components. Examination of the C^{14} profile suggests that the shoulder on the leading edge of the main peak could be due to another component of about 25S. By analogy to the normal sequence of ribosome synthesis this could be identified as the neosome second stage precursor known to have a sedimentation coefficient of somewhat less than 30 (Paper III). It can also be identified as the 24S particle observed by Nomura and Watson (1959) in cell extracts after prolonged chloramphenicol inhibition.

D. DISCUSSION

1. *The Assembly of the Ribosomes.* The experiments on the synthesis of ribosomal protein do not add any fundamentally new features to the sequence of ribosome synthesis based on studies of RNA synthesis (Paper III). They do, however, confirm previous indications that some of the steps in the sequence represent the addition of protein. The fact that a high proportion of the leucine label enters 30S and 50S ribosomes directly with delays of less than one minute shows that the last stage, 43S neosome \rightarrow 50S ribosome, involves the addition of protein. This is in agreement with the fact that no new RNA is incorporated directly into ribosomes and with the low protein/RNA ratio of neosomes indicated by their column behavior.

One other feature is clear from the leucine pulse and chase experiments. Since the 43S receives some label at early times, it is evident that not all the protein of the 50S is added in one step. In fact the chase experiment shows that the 50S increases some 30 per cent in specific radioactivity during an 8 minute chase period at the expense of the 43S. Rough estimates made from the two leucine experiments suggest that the 43S has a protein/RNA ratio one-quarter to one-third that of the 50S ribosome.

The final flow diagram, including both the RNA and protein moieties of ribosomes, is shown in Fig. 6. The open and shaded areas are proportional to RNA and protein contents. The eosome is shown as pure RNA since there are no measurements of its protein content and it must certainly be small. There is evidence of excess leucine radioactivity at early times (Fig. 1(a) and (b)) in the 14S region but the significance of this is not clear. The 30S neosome is shown with less than half of the protein of the 30S ribosome by analogy to the 43S neosome. However,

its protein content cannot be measured because of the lack of resolution between objects in the 30S size range. The 30S neosome which is precursor to the 43S is shown as a separate object from that leading to the 30S ribosomes, which may well be an unnecessary complication.

The 43S neosome is shown with only one-quarter of the protein of the 50S ribosome. This quantity is uncertain and it is not known what fraction of its protein enters directly in the formation of the 43S or by way of the 30S precursor to it. While these estimates of relative protein contents in neosome and ribosome are

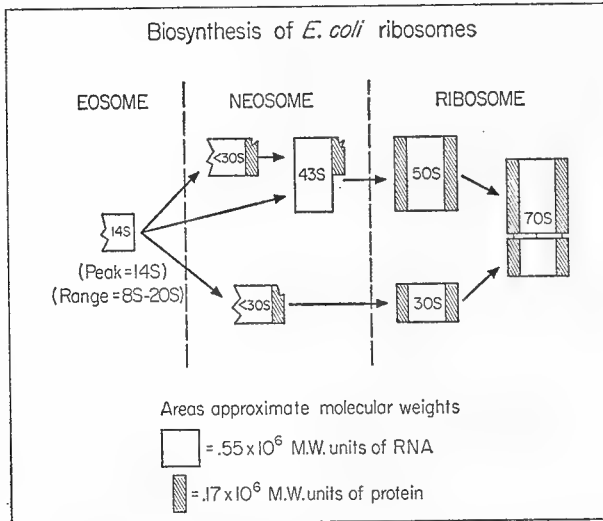


FIGURE 6 The biosynthesis of ribosomes in *E. coli*. The open and shaded areas are proportional to weights of RNA and protein respectively.

crude and preliminary, it is clear that the conversion of neosome to ribosome involves only the addition of protein and that the greater part of the ribosomal protein is added in this step.

The effect of chloramphenicol is just what one would expect from this model. The predominant effect is the accumulation of what appears to be eosome material by both sedimentation and column analysis. Moreover the nucleotide composition of this material is like that of ribosomes (Bolton, 1959). Apparently chloramphenicol inhibits the addition of protein to eosomes. The minor peak appearing at about 25S may represent small quantities of neosome produced in the presence of chloramphenicol since even at 200 $\mu\text{g/ml}$ the inhibition of protein synthesis is not complete.

One feature of the general sequence of ribosome synthesis must be emphasized. The addition of RNA and protein occur in time-separated stages. This is clear from the very observation of RNA-rich intermediates.

2. *Role of Ribosome Synthesis in Replication.* Ribosomes are ubiquitous,

appear in greatest concentration where or when protein synthesis occurs, and newly synthesized protein is found in association with them. Furthermore, the rate of protein synthesis depends, not on the rate of RNA synthesis, but on the number of ribosomes present (Kjelgaard, 1961). Thus it seems likely that the steps in ribosome synthesis are relevant to the process of replication but the exact correlation with other parts of this process is difficult to discern. For example, is the synthesis of the ribosome directed by DNA or autocatalytic? Is the process of ribosome synthesis related to the transfer of information? At this time no exact answers can be given to these questions. There is, however, information which justifies speculation and suggests future experiments.

In the high concentrations of magnesium, a large fraction of the eosome is found in association with 70S and larger ribosomes. This observation is open to four interpretations. First, this association may be meaningless due to simple adsorption of eosome on the large ribosomes. Second, it may be due to the existence of large, newly formed particles which are broken down to eosomes and neosomes when magnesium concentration is reduced. Third, eosomes may actually have been synthesized in association with the 70S ribosomes. Finally, the eosomes may have been transferred from their sites of synthesis to the large ribosomes, perhaps in order to carry out a role in information transfer.

The third and fourth of these alternatives have the most theoretical interest and may well represent different views of the same process. Both these alternatives are consistent with the experiments of Brenner, Meselson, and Jacob (1961) if it is assumed, in the case of bacteriophage infection, that ribosome synthesis is effectively halted at the eosome stage.

The third alternative gains some support from the autocatalytic rate of ribosome synthesis which occurs when cells recover from magnesium starvation (McCarthy, 1962). The fourth alternative is supported by the observation of Caro and Forro (1961) that newly synthesized RNA shows the same localization as does DNA. Thus the eosomes may be formed in association with DNA and subsequently removed from the DNA by transfer to association with a ribosome. Consequently the rate of synthesis might be limited, both by the quantity of DNA acting as template, and by the number of ribosomes available to strip the template. The picture of the process is highly speculative and there is no information whatever on the location of other events such as the addition of protein.

The eosome might act as template for protein synthesis for the period before it is covered by other RNA and protein to become a finished ribosome. Its lifetime in the eosome stage does correspond to the lifetime of the enzyme-forming unit for β -galactosidase (Pardee and Prestidge, 1961; Boezi and Cowie, 1961). This correspondence, however, is between the average lifetime of all eosomes and the lifetime of one particular enzyme-forming unit. There may well be a broad distribution of eosome lifetime which would allow some protein-synthesizing units to act

for prolonged periods without replenishment of the template. Reticulocytes are able to synthesize a particular protein (hemoglobin) for long periods when DNA is apparently absent from the cell and RNA synthesis occurs at very low rates (Kruh and Borsook, 1956; Nathans *et al.*, 1961).

While it is clear from kinetic studies (Paper III) that the majority of the material in the eosome fraction must be considered ribosome precursor, it is possible that other types of RNA molecules in this size range are also present. For example it appears that in the case of bacteriophage infection, there is synthesized an RNA molecule which reflects the nucleotide composition of the infecting DNA and is able to form hybrid double strands with it (Hall and Spiegelman, 1961). In addition, the presence of RNA molecules with similar properties in uninfected cells can be demonstrated by growth under special conditions (Hayashi and Spiegelman, 1961). The kinetic study reported in Paper III, Fig. 7, suggests that there does exist a small component of very rapidly labeled RNA more closely associated with the 70S ribosome than the majority of the eosome.

In view of the complexity of the processes of RNA synthesis in the growing cell, rapid labeling cannot be considered to demonstrate turnover (in the sense of synthesis and degradation), nor does association with large ribosomes prove an object's role as informational RNA (Gros *et al.*, 1961). If indeed a distinction does exist between the eosome (considered as ribosome precursor) and messenger (considered as rapidly turning over RNA) new evidence is needed in order to assess their relative quantities and roles in the growing cell. Further attempts at fractionation, measurements of nucleotide compositions and ability to specifically hybridize with DNA may resolve the question.

In any event the eosomes, or a fraction of them, seem to be likely candidates for template material. There is as yet no binding evidence which requires the existence of an RNA other than that normally destined to be ribosomal RNA to carry out the function of information transfer from the DNA to the sites of protein synthesis.

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III.B.4 The Nucleotide Base Composition of Ribonucleic Acid from Several Microbial Species

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SUMMARY

The nucleotide base compositions of alkali digests of various microbial RNA components have been measured by an isotope dilution technique and column chromatography. All bacterial samples for analysis were taken during the exponential growth of cultures in a simple chemically defined medium. The total trichloroacetic acid-precipitable RNA, the 30-S, 50-S and 70-S ribonucleoprotein particles, and the soluble RNA have been measured in each of five bacterial species. The corresponding fractions in yeast have also been analyzed. In addition, the base composition of the rapidly labeled 14-S RNA fraction has been measured after short periods of ^{32}P incorporation into bacteria. Only in this fraction has any consistent correlation between RNA composition and the DNA composition of the bacterial species been found.

INTRODUCTION

The RNA of bacteria is remarkably invariable in nucleotide base composition, whilst the DNA nucleotide composition may vary widely from species to species. BELOZERSKY AND SPIRIN¹, on the basis of determinations of the total unfractionated cell RNA in many species, indicated that there might be a slight correlation between the composition of the unfractionated RNA and the DNA. MIURA² has reported the finding of a similar slight correlation in the s-RNA within a group of six bacteria. Other workers have reported no^{2,3} or a very marginal⁴ correlation between the base composition of the ribosomal RNA in bacteria and the DNA. No compositional differences have been observed^{3,5} in the compositions of the 16-S and the 23-S RNA particles resulting from the phenol treatment of *E. coli* 70-S ribosomes.

There may exist a small fraction of the bacterial RNA which possesses a base composition like that of the DNA, uracil substituting for thymine⁶. Reinforcing this possibility, the composition of newly formed RNA in bacteria and in yeast, as measured by the short exposure of cultures to [^{32}P]orthophosphate, bears some resemblance to the DNA of the organisms^{7,8}. However, in no case as yet has the composition of such fractions been reported to be identical with that of the DNA in steadily growing

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cells. HAYASHI AND SPIEGELMAN⁹ have reported the formation of an RNA with the base composition of the cell DNA in bacteria immediately after transfer of cells from broth to glucose media. The presence of an RNA fraction corresponding to infecting bacteriophage DNA has been reported during the infection of *E. coli* by T-2 bacteriophage^{10,11}. A similar RNA fraction corresponding to T-4 bacteriophage has been purified from infected cells of *E. coli* (see ref. 12).

The bulk RNA of bacteria has at most a very small correlation with the DNA of the species. In *E. coli*, SPAHR AND TISSIÈRES⁵ have reported small differences in the nucleotide base composition of the 30-S and the 50-S ribonucleoprotein particles. BOLTON¹³ has also found compositional differences in these fractions and has shown differences in the oligonucleotide pattern resulting from digestion of the 30-S and 50-S particles with pancreatic ribonuclease. Differences in elution by NaCl from DEAE-cellulose columns have been observed for phenol-extracted RNA from *E. coli* 30-S and 50-S particles¹⁴.

In the present work, five bacterial species and one of yeast have been examined to see if any consistent correlation between the RNA and the DNA base composition exists in one or more of the RNA fractions which could be isolated. The technique of isotope dilution was used to determine the composition of these RNA fractions with the greatest possible accuracy, so that even fairly small differences in base composition could be detected amongst the fractions of a given species.

METHODS

The bacterial species *Pseudomonas aeruginosa* A.T.C.C. 9027, *Aerobacter aerogenes* A.T.C.C. 211, *Escherichia coli* ML 30, *Bacillus subtilis* A.T.C.C. 6051, and *Proteus vulgaris* A.T.C.C. 4669 were used. The yeast used was *Saccharomyces cerevisiae* A.T.C.C. 2338. The DNA (guanylic acid+cytidylic acid)/(adenylic acid+thymidylic acid) ratios of these organisms lie in a range from 1.75 (*Pseudomonas*) to 0.6 (*Proteus* and *Saccharomyces*)¹. All bacterial cultures were grown in aerated media at 37° containing 0.01 M Tris adjusted to pH 7.2 with HCl, 0.01 M Na₂SO₄, 0.01 M MgCl₂, NH₄Cl, 5 g/l, and sodium and potassium phosphates to give a concentration of 0.0002 M with respect to PO₄³⁻. Yeast was grown in a medium containing 1 % (w/v) bacto-peptone, 0.1 % (w/v) yeast extract, 0.01 M MgCl₂, 0.01 M Na₂SO₄, 5 % (w/v) glucose, and 1 g/l each of NaCl and KCl. The cultures were aerated and grown at 30°.

For the production of RNA for base-composition analyses, the bacteria and yeast were grown in the presence of [³²P]orthophosphate for several hours in the logarithmic phase. They were then harvested at cell densities of about 1 g/l and were washed three times in 0.01 M Tris buffer (pH 7.3) containing 0.01 M MgCl₂. The pellet was resuspended in the Tris-MgCl₂ buffer and the cells were broken in the French pressure cell at 15 000 lb/in². The cell extract was then centrifuged at 105 000 × g for 2 min to remove cell walls and unbroken cells. The supernatant was further centrifuged at 105 000 × g for 45 min to pellet the 70-S ribosomes. The pellet was then washed with 0.01 M Tris-0.01 M MgCl₂ buffer (pH 7.3), resuspended and repelleted by a further centrifugation for 45 min. In this way a purified sample of 70-S ribosomes was prepared. *E. coli* ML 30 unlabeled 70-S ribosomes were also prepared from one batch of cells by the same method.

30-S and 50-S ribosomes derived from the 70-S particles were purified by the use of the sucrose density-gradient sedimentation method¹⁵. A small quantity of

labeled 70-S ribosomes (less than 0.1 mg) was suspended in 0.0001 *M* MgCl_2 -Tris buffer, and was centrifuged at 37 000 rev./min in the swinging bucket rotor for 160 min, through a 5–20 % (w/v) sucrose density gradient containing 0.0001 *M* MgCl_2 -Tris buffer. At this RNA concentration, the resolution of the 30-S and 50-S ribosomes was sufficient to allow samples to be taken without cross-contamination (Fig. 1).

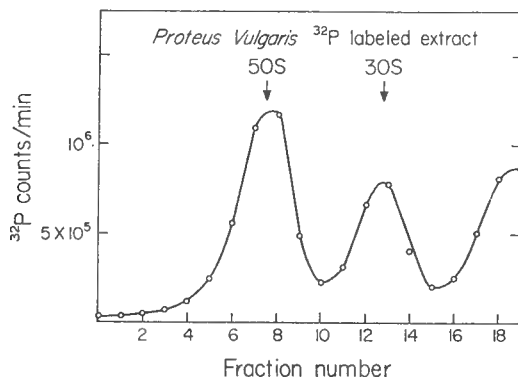


Fig. 1. Sucrose density-gradient sedimentation pattern of ^{32}P -labeled 30-S and 50-S ribosomes of *P. vulgaris*. Sucrose concentration 5–20 % in 0.0001 *M* MgCl_2 -Tris buffer. Centrifugation at 37 000 rev./min for 160 min at 4°.

s-RNA was purified by further centrifugation of the bacterial extract from which the 70-S particles had been removed (240 min at 105 000 $\times g$). The supernatant was carefully pipetted off and was then treated with phenol and 2 % sodium dodecyl sulphate¹⁶ after the manner of KIRBY¹⁷. After precipitation by 3 vol. of cold 95 % ethanol, the s-RNA was dissolved in 0.01 *M* Tris–0.01 *M* MgCl_2 buffer (pH 7.3) and was adsorbed on DEAE-cellulose. It was then eluted in a linear NaCl gradient (0.2–1.0 *M*)¹⁸. s-RNA eluted at 0.5 *M* NaCl, and any degraded ribosomal RNA not pelleted by centrifugation eluted at 0.8–1.0 *M* NaCl (Fig. 2).

Unfractionated cell RNA was obtained by precipitating labeled cells in cold 5 % (w/v) trichloroacetic acid solution and filtering off the material on Millipore filters¹⁹.

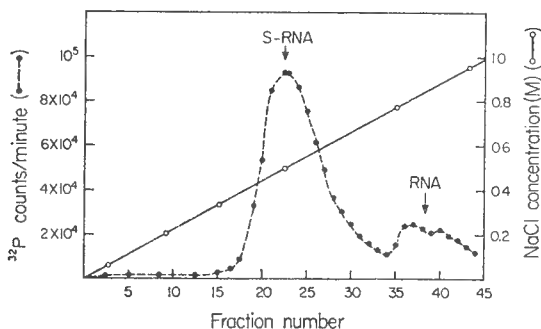


Fig. 2. Elution by NaCl from a DEAE-cellulose column of phenol-treated supernatant of ^{32}P -labeled *B. subtilis* cell extract, obtained after centrifugation at 105 000 $\times g$ for 240 min. Linear gradient of NaCl (0.2–1.0 *M*) in 0.01 *M* Tris–0.01 *M* MgCl_2 buffer (pH 7.3). ●—●, ^{32}P counts/min; ○—○, NaCl concentration, *M*.

The rapidly labeled 14-S RNA component was isolated by several methods to be described later.

All samples to be analyzed were precipitated by cold 5 % trichloroacetic acid and filtered before alkaline hydrolysis was carried out. Repeated washings of the filter with 5 % trichloroacetic acid effectively removed contaminating 5'-nucleotides arising from the pool of RNA precursors in the cells.

Hydrolysis of the RNA samples was carried out by treatment of the filters with 0.38 *M* KOH for 15 h at 37°. Excess unlabeled *E. coli* ML 30 70-S RNA, prepared by phenol treatment of 70-S ribosomes, was routinely added. The soluble brown material produced by the dissolution of the filter in the alkali did not interfere with the analyses. Excess alkali was neutralized by 1.0 *M* perchloric acid and the resulting precipitate was centrifuged. The 2'- and 3'-nucleotides were adsorbed on a 0.5 × 15 cm Dowex-1-formate column (200–400 mesh)²⁰ and elution was effected by a non-linear gradient²¹ of formic acid (0–4 *M*)²² so that the 2'- and 3'-isomers of adenylic and guanylic acids were partially resolved. In this way, a check was kept on the hydrolytic procedure and on 5'-nucleotide contaminations, by a comparison of the specific activities of the 2'- and 3'-isomers. No compositions have been quoted in this paper in which any differences in 2'- and 3'-nucleotide specific activities occur.

The technique of isotope dilution allowed the minimization of some of the more likely errors in base-composition determination when simple summation of nucleotide absorbancies or ³²P counts are used for base-analysis measurements. The sample of unlabeled RNA was used to supply effectively all the ultraviolet absorbancy of the eluted 2'- and 3'-nucleotides, and many determinations of the ratio of radioactivity of the sample to ultraviolet absorption were made for each nucleotide in each analysis, contaminating material being readily detected. Further, inaccuracies in the determinations due to incomplete digestion of the RNA, to selection of some of the nucleotides during the mechanics of transfer and to possible nucleotide interconversions or dephosphorylations during hydrolysis or preparation of the RNA fractions are greatly minimized. However, for accurate absolute determinations of the nucleotide base compositions of the labeled samples, the base composition of the *E. coli* 70-S RNA used as unlabeled carrier must be accurately determined. The accuracy of this determination does not affect the relative compositions of any two or more labeled samples.

RESULTS

Analysis of the standard E. coli RNA

The composition of the single batch of *E. coli* ML 30 70-S RNA used as unlabeled carrier in all subsequent determinations was measured by alkaline hydrolysis of a sample, column chromatography and summation of the ultraviolet absorbancies obtained from the elution of each nucleotide being used. In the digests, approx. 98 % of the material hydrolyzed was recovered from the column. These measurements were checked against the result obtained by the summation of the ³²P counts/min contained in each nucleotide after hydrolysis and column chromatography of a labeled sample of *E. coli* 70-S RNA, prepared in the same way. Finally, to check the validity of the absorbancy coefficients used in the calculations of base composition throughout, labeled *E. coli* RNA was hydrolyzed by alkali in the presence of an excess of unlabeled material. All fractions collected were acidified to pH 2.0 with 0.1 *M* HCl

before determining the ultraviolet absorbancy. Readings of absorbancy in the range 256–280 $m\mu$ were made on a Zeiss spectrophotometer, and specific activities were measured only in those fractions having absorbancies of between 0.8 and 3.0 at the wavelength of maximum absorbancy for each nucleotide at pH 2.0. Using the millimolar extinction coefficients at pH 2.0: cytidylic acid, 13.0 at 280 $m\mu$; adenylic acid, 15.1 at 257 $m\mu$; guanylic acid, 12.2 at 256 $m\mu$; uridylic acid, 10.0 at 262 $m\mu$, the specific activities of the nucleotides were found to be constant to within 1 %.

The possibility of the fractionation of the standard RNA by the phenol procedure was also checked by comparison of the composition determined from phenol-extracted 70-S RNA and from trichloroacetic acid-precipitated ^{32}P -labeled 70-S ribosomes of *E. coli*. No significant differences could be detected.

The composition of bulk RNA components in the cell

Table I indicates the nucleotide base composition of the *E. coli* ML 30 70-S RNA as determined by two methods. The results are the mean of several determinations by each method.

TABLE I

DETERMINATIONS OF THE BASE COMPOSITION OF *Escherichia coli* 70-S RNA

Several determinations by each of the two methods were carried out. The mean nucleotide base composition used in experiments was: cytidylic acid, 21.9 mole %, adenylic acid, 25.1 mole %, guanylic acid, 32.6 mole %, uridylic acid, 20.4 mole %. All nucleotide base-composition analyses are accurate to ± 1.5 %.

Nucleotide	Summation of ^{32}P counts in nucleotides (mole %)	Summation of ultraviolet absorbancies of nucleotides at pH 2 (mole %)
Cytidylic acid	21.7	22.0
Adenylic acid	25.2	25.1
Guanylic acid	32.8	32.4
Uridylic acid	20.3	20.5

The base compositions of the unfractionated cell RNA precipitable by cold 5 % trichloroacetic acid, the 70-S, 50-S and 30-S ribosomes, and the s-RNA in the five bacterial species are given in Tables II–VI. In comparison, the base composition of the 80-S, 60-S and 40-S ribosomes of yeast, and the s-RNA is given in Table VII. The slight differences observed in the 30-S and the 50-S ribosomes nucleotide base composition in a given species are reproducible to better than 1 %. As, in several of the determinations, the compositions of the RNA in the 50-S and 30-S particles differ in individual nucleotides by as much as 10–15 % in a single species, these differences are probably real. Neither the unfractionated cell RNA, the 70-S RNA, nor the s-RNA were found to possess a definite correlation with the DNA for any species. In fact, the compositions of these fractions in the five bacterial species are all invariable within the limits of the experimental error of determination. Yeast has a ribosomal RNA and total-cell RNA base composition basically unlike that of bacteria. The results for yeast ribosomal and s-RNA can be compared with those of MONIER, STEPHENSON AND ZAMECNIK²³. If there exists in these fractions an RNA with a composition like that of the DNA, the accuracy of measurement by the isotope dilution technique cannot permit it to be more than 10 % of the RNA.

TABLE II

COMPOSITIONS OF RNA FRACTIONS OF *Pseudomonas aeruginosa* ATCC 9027

DNA composition: adenylic acid = thymidylic acid, 18 mole %; guanylic acid = cytidylic acid, 32 mole %. The underlined values in the 30-S and 50-S base-composition analyses are those which are different in the two subunits from the bacterial species. All nucleotide base-composition analyses are accurate to ± 1.5 %.

Nucleotide	Total RNA	70-S	50-S	30-S	s-RNA
Cytidylic acid	22.2	21.7	21.2	21.6	28.3
Adenylic acid	25.7	25.7	<u>26.3</u>	<u>25.1</u>	20.8
Guanylic acid	31.3	31.6	<u>31.2</u>	<u>32.8</u>	33.8
Uridylic acid	20.8	21.0	21.3	20.5	17.1
Purine					
Pyrimidine	1.33	1.35	1.35	1.36	1.20
Guanylic acid + cytidylic acid					
Adenylic acid + uridylic acid	1.15	1.14	1.10	1.19	1.64

TABLE III

COMPOSITION OF RNA FRACTIONS OF *Aerobacter aerogenes* ATCC 211

DNA composition: adenylic acid = thymidylic acid, 22 mole %; guanylic acid = cytidylic acid, 28 mole %. The underlined values in the 30-S and 50-S base-composition analyses are those which are different in the two subunits from the bacterial species. All nucleotide base-composition analyses are accurate to ± 1.5 %.

Nucleotide	Total RNA	70-S	50-S	30-S	s-RNA
Cytidylic acid	22.6	21.9	22.0	22.4	29.2
Adenylic acid	25.0	25.5	25.6	25.3	19.7
Guanylic acid	31.7	31.5	31.2	30.8	32.3
Uridylic acid	20.7	21.1	21.2	21.5	18.8
Purine					
Pyrimidine	1.32	1.33	1.32	1.27	1.10
Guanylic acid + cytidylic acid					
Adenylic acid + uridylic acid	1.19	1.15	1.14	1.15	1.60

TABLE IV

COMPOSITIONS OF RNA FRACTIONS OF *Escherichia coli* ML 30

DNA composition: adenylic acid = thymidylic acid, 24 mole %; guanylic acid = cytidylic acid, 26 mole %. The underlined values in the 30-S and 50-S base-composition analyses are those which are different in the two subunits from the bacterial species. All nucleotide base-composition analyses are accurate to ± 1.5 %.

Nucleotide	Total RNA	70-S	50-S	30-S	s-RNA
Cytidylic acid	22.1	21.9	<u>21.5</u>	<u>22.7</u>	29.5
Adenylic acid	25.2	25.1	25.4	24.8	19.7
Guanylic acid	32.5	32.6	<u>33.5</u>	<u>31.0</u>	33.8
Uridylic acid	20.2	20.4	<u>19.6</u>	<u>21.5</u>	17.0
Purine					
Pyrimidine	1.37	1.36	1.44	1.26	1.17
Guanylic acid + cytidylic acid					
Adenylic acid + uridylic acid	1.20	1.20	1.22	1.16	1.71

TABLE V

COMPOSITION OF RNA FRACTIONS OF *Bacillus subtilis* ATCC 6051

DNA composition: adenylic acid = thymidylic acid, 29 mole %; guanylic acid = cytidylic acid, 21 mole %. The underlined values in the 30-S and 50-S base-composition analyses are those which are different in the two subunits from the bacterial species. All nucleotide base-composition analyses are accurate to $\pm 1.5\%$.

Nucleotide	Total RNA	70-S	50-S	30-S	s-RNA
Cytidylic acid	22.1	22.3	22.5	22.3	28.3
Adenylic acid	25.5	25.9	26.5	26.5	20.2
Guanylic acid	31.4	31.0	<u>32.0</u>	<u>29.6</u>	33.9
Uridylic acid	21.0	20.8	<u>19.3</u>	<u>21.6</u>	17.6
Purine					
Pyrimidine	1.32	1.32	1.39	1.28	1.17
Guanylic acid + cytidylic acid					
Adenylic acid + uridylic acid	1.17	1.15	1.20	1.08	1.65

TABLE VI

COMPOSITION OF RNA FRACTIONS OF *Proteus vulgaris* ATCC 4669

DNA composition: adenylic acid = thymidylic acid, 31 mole %; guanylic acid = cytidylic acid, 19 mole %. The underlined values in the 30-S and 50-S base-composition analyses are those which are different in the two subunits from the bacterial species. All nucleotide base-composition analyses are accurate to $\pm 1.5\%$.

Nucleotide	Total RNA	70-S	50-S	30-S	s-RNA
Cytidylic acid	22.6	21.7	<u>21.3</u>	<u>23.0</u>	29.3
Adenylic acid	24.6	26.2	<u>26.5</u>	<u>24.7</u>	19.1
Guanylic acid	32.0	31.4	<u>31.4</u>	<u>31.9</u>	33.3
Uridylic acid	20.8	20.7	20.8	20.4	18.3
Purine					
Pyrimidine	1.30	1.35	1.37	1.30	1.11
Guanylic acid + cytidylic acid					
Adenylic acid + uridylic acid	1.21	1.13	1.11	1.22	1.67

TABLE VII

COMPOSITIONS OF RNA FRACTIONS OF *Saccharomyces cerevisiae*

DNA composition: adenylic acid = thymidylic acid, 32 mole %; guanylic acid = cytidylic acid, 18 mole %. The underlined values in the 40-S and 60-S base-composition analyses are those which are different in the two subunits from the bacterial species. All nucleotide base-composition analyses are accurate to $\pm 1.5\%$.

Nucleotide	Total RNA	80-S	60-S	40-S	s-RNA
Cytidylic acid	19.4	19.2	19.0	19.1	26.3
Adenylic acid	26.8	27.2	<u>27.9</u>	<u>25.2</u>	19.2
Guanylic acid	28.3	28.2	<u>28.4</u>	<u>28.4</u>	34.3
Uridylic acid	25.5	25.4	<u>24.7</u>	<u>27.3</u>	20.2
Purine					
Pyrimidine	1.23	1.24	1.29	1.15	1.15
Guanylic acid + cytidylic acid					
Adenylic acid + uridylic acid	0.91	0.90	0.90	0.91	1.55

The composition of the 14-S RNA fraction

It has been established that the first detectable labeled polynucleotide material formed during the incorporation of [³²P]- or [¹⁴C]uracil into bacterial RNA has different sedimentational and chromatographic properties from the RNA detectable by ultraviolet absorption²⁴. It has also been found that most of the [¹⁴C]uracil which is incorporated into this fraction is eventually incorporated into the RNA of the ribosomes^{25,26}. MCCARTHY, BRITTEN AND ROBERTS^{25,26} have termed this fraction the "eosome". As this material accounts for effectively all the ³²P-labeled RNA present in short periods of isotope incorporation, its base composition should be similar to that of unfractionated cells at these times.

The five species of bacteria used in the bulk RNA studies above were exposed to short periods of [³²P]orthophosphate incorporation during exponential growth. The cells were then squirted into 10 % cold trichloroacetic acid and filtered on Millipore filters. Many washes of trichloroacetic acid were given to remove most of the 5'-nucleotides on the filter. From an aliquot of cells which had been poured onto crushed ice rather than into trichloroacetic acid, 14-S RNA was then isolated. The analyses of the pulse-labeled RNA in the five species are given in Table VIII.

The extracts from the cells poured onto crushed ice were adsorbed on DEAE-cellulose and eluted by a linear NaCl gradient of 0.2–1.0 M NaCl in 0.01 M Tris–0.01 M MgCl₂ buffer (pH 7.3). Fig. 3 shows a typical elution pattern. It can be seen

TABLE VIII
COMPOSITIONS OF LABELED RNA FORMED DURING SHORT EXPOSURE OF
BACTERIA TO [³²P]ORTHOPHOSPHATE
All nucleotide base-composition analyses are accurate to ± 1.5 %.

Species	Time of labeling with isotope (min)	Labeled RNA composition (trichloroacetic acid-precipitable) (mole %)				Guanylic acid + cytidylic acid Adenylic acid + uridylic acid
		Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid	
<i>Ps. aeruginosa</i>	4	25.4	21.1	31.9	21.6	1.34
<i>A. aerogenes</i>	4	23.4	24.8	30.3	21.5	1.16
<i>E. coli</i>	2	22.9	25.0	29.5	22.6	1.10
<i>B. subtilis</i>	2	23.3	25.6	27.7	23.4	1.04
<i>P. vulgaris</i>	4	22.2	26.7	27.0	24.1	0.97

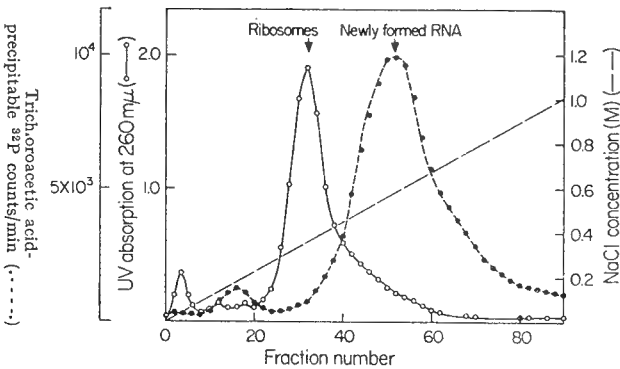


Fig. 3. Elution by NaCl from a DEAE-cellulose column of a cell extract from an *E. coli* culture labeled for 3 min by ³²P. Linear NaCl gradient (0.2–1.0 M) in 0.01 M Tris–0.01 M MgCl₂ buffer (pH 7.3). ---, NaCl concentration, M (●-●), trichloroacetic acid-precipitable ³²P counts/min; ○-○, ultraviolet absorption at 260 mμ.

that only one labeled component, not tracking with any of the ultraviolet-absorbing material, elutes at 0.6 *M* NaCl. This material was pooled, trichloroacetic acid precipitated and collected by filtration. Analysis of the filters gave the compositions listed in Table IX. In each of the species examined, the base composition of the 14-S or "eosome" obtained in this way is identical within experimental error to that of the total-cell labeled RNA at this time.

TABLE IX
COMPOSITION OF THE 14-S (EOSOME) RNA COMPONENT OF BACTERIA
PURIFIED BY DEAE-CELLULOSE CHROMATOGRAPHY

All nucleotide base-composition analyses are accurate to $\pm 1.5\%$.

Species	Time of labeling with isotope (min)	14-S RNA composition				Guanylic acid+cytidylic acid Adenylic acid+uridylic acid
		Cytidylic acid	Adenylic acid	Guanylic acid	Uridylic acid	
<i>Ps. aeruginosa</i>	4	25.6	20.8	31.7	21.9	1.31
<i>E. coli</i>	2	22.7	25.1	29.1	23.1	1.07
<i>B. subtilis</i>	2	22.5	25.3	28.0	24.2	1.02
<i>P. vulgaris</i>	4	21.9	27.0	27.6	23.5	0.98

A culture of *B. subtilis* was given a 3-min labeling period with ^{32}P during exponential growth. The base composition of the total-cell labeled RNA was measured, and a sample of the cell juice was treated with phenol, and after alcohol precipitation and dissolving the RNA in 0.01 *M* Tris-0.01 *M* MgCl_2 buffer (pH 7.3), it was then adsorbed on a methylated serum albumin coated kieselguhr column²⁷. The RNA was eluted by a linear gradient of NaCl from 0.4-1.1 *M* in 0.04 *M* phosphate buffer (pH 6.7). The elution pattern is shown in Fig. 4.

The labeled RNA does not track exactly with the 16-S and 23-S RNA produced from the bulk of the RNA components of the cell. There are three radioactive peaks, but analysis of each showed that there was no difference in base compositions of any

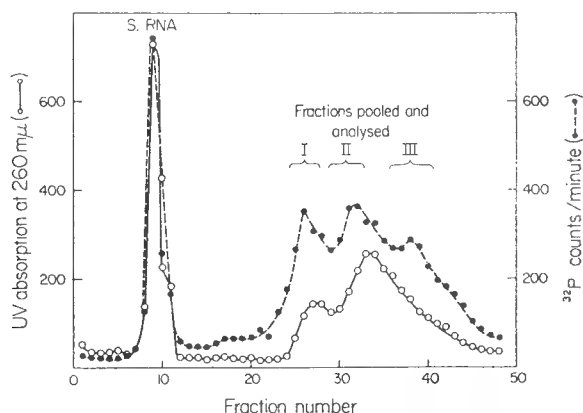


Fig. 4. Elution by NaCl from a methylated serum albumin coated kieselguhr column of a cell extract from an *B. subtilis* culture labeled for 3 min by ^{32}P . The cell extract was treated with phenol to remove protein from the ribosomes before adsorption on the column. Linear gradient of NaCl (0.4-1.1 *M*) in 0.04 *M* potassium phosphate buffer, pH 6.7. ●-●, trichloroacetic acid-precipitable ^{32}P counts/min; ○-○, ultraviolet absorption at 260 *mμ*.

one peak from the composition of the material eluted at 0.6 *M* NaCl from DEAE-cellulose or from the total-cell labeled RNA at this time. It is evident that under these conditions no further fractionation of the newly formed RNA labeled with ^{32}P has been achieved.

The 14-S component of *E. coli* labeled for 3 min by ^{32}P was isolated by sucrose density-gradient centrifugation in the swinging bucket. After centrifugation at 37 000 rev./min for 160 min a peak sedimenting at about 14 S was clearly resolved by its radioactivity (Fig. 5). This peak was collected and trichloroacetic acid precipitated.

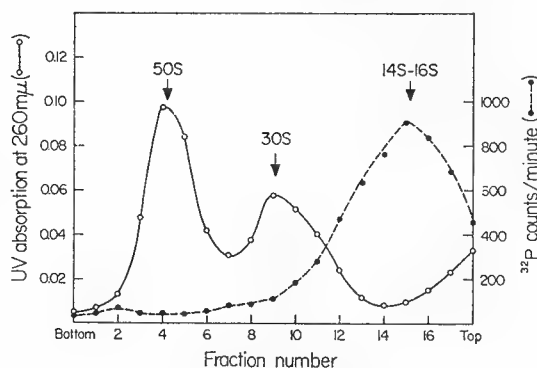


Fig. 5. Sucrose density-gradient sedimentation of a cell extract from *E. coli* labeled for 3 min by ^{32}P . Sucrose concentration 5–20 % in 0.0001 *M* MgCl_2 -Tris buffer. Centrifugation at 37 000 rev./min for 160 min at 4°. ○-○, ultraviolet absorption at 260 mμ; ●-●, trichloroacetic acid-precipitable ^{32}P counts/min.

Its analysis showed that it was identical to the total-cell labeled RNA at this time, and to the material eluted from DEAE-cellulose at 0.6 *M* NaCl. This would indicate that the eosome or 14-S RNA can be isolated as a discrete object without measurable change in base composition and that column chromatography either by the MANDELL AND HERSHEY column²⁷ of phenol-treated RNA, or by DEAE-cellulose of untreated cell extracts, does not result in the isolation of newly formed RNA with a base composition any different from that obtained by trichloroacetic acid precipitation of unfractionated labeled cells.

DISCUSSION

The analyses of the bulk RNA components in the five species of bacteria used indicate no obvious relationship in the nucleotide base composition of the various purified RNA fractions to the DNA. The composition of the RNA which comprises most of this material in the cells, the 70-S ribosomes, is extremely invariable in composition from species to species. The s-RNA also appears to be very constant in composition (Fig. 6). The composition obtained for purified *E. coli* s-RNA agrees with the results obtained by DUNN, SMITH AND SPAHR²⁸ and by ZILLIG *et al.*²⁹.

Subfractionation of the 70-S component of bacteria into 50-S and 30-S or of yeast 80-S into 60-S and 40-S has brought to light some differences in base composition of the two fractions. In general, purine contents are higher, pyrimidines lower in the larger (50-S or 60-S) than in the smaller (30-S or 40-S) ribosomal subunits. There is, however, no uniformly consistent relationship in composition between

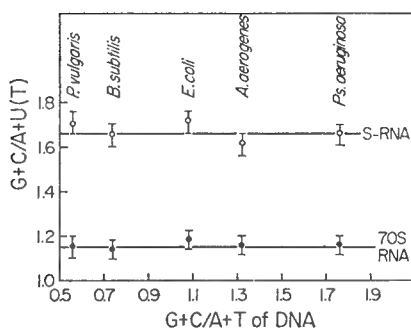


Fig. 6. Comparison of (guanylic acid + cytidylic acid)/(adenylic acid + uridylic acid) values for s-RNA and 70-S RNA, from bacteria with DNA (guanylic acid + cytidylic acid)/(adenylic acid + thymidylic acid) values ranging from 0.6 to 1.75. ○—○, s-RNA values; ●—●, 70-S ribosomal RNA values.

the DNA, and either 30-S or 50-S in the bacterial species. The weak relationship between the DNA and s-RNA in bacteria reported by MIURA² is possibly due to contamination of the soluble fraction by the 14-S RNA component stripped from the 70-S ribosomes during washing in 0.14 M NaCl.

The 14-S RNA component of bacterial cells has been detected by short periods of [¹⁴C]uracil incorporation into growing cells of *E. coli*^{25,26,30,31}. It has been variously ascribed the role of "messenger" RNA^{30,31} and of "ribosomal RNA precursor"^{25,26}. By present theories, these two roles would predict base compositions of the 14-S component of two types. The "messenger" theory, assuming that the RNA carries genetic information, postulates that the base composition of this RNA fraction is like that of the DNA in the cell, whilst the "ribosomal precursor" theory^{25,26} would predict the composition to be like that of the ribosomes.

The 14-S fraction has been found to be very different in base composition from the normal total trichloroacetic acid-precipitable RNA in the cell or from the ribosomes. Figs. 7 and 8 indicate a possible relationship between the DNA composition of the bacteria, the 14-S RNA and 70-S RNA base compositions in each of the five

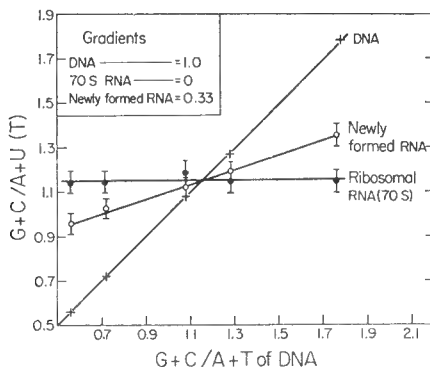


Fig. 7. Comparison of (guanylic acid + cytidylic acid)/(adenylic acid + uridylic acid (thymidylic acid)) values for 70-S ribosomal RNA, 14-S RNA and DNA from bacteria with DNA (guanylic acid + cytidylic acid)/(adenylic acid + thymidylic acid) values ranging from 0.6 to 1.75. +—+, DNA values; ○—○, newly formed (14-S) RNA values; ●—●, 70-S ribosomal RNA values.

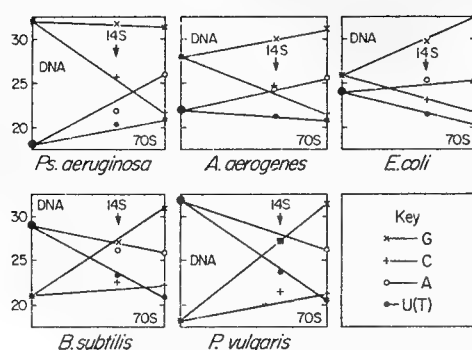


Fig. 8. Graphical representation of nucleotide base composition of DNA, 14-S RNA and 70-S ribosomal RNA in each of the five bacterial species used. Ordinate, nucleotide base composition (mole %). On the left ordinate of each graph, DNA base composition, on the right ordinate, 70-S RNA base composition. 14-S RNA composition as best fit between these two compositions.

species. It can be seen (Fig. 7) that if the 14-S fraction were in fact composed of two entities of RNA with different base compositions corresponding to either the DNA or to the ribosomal RNA, then in each case the 14-S RNA would be made up of approx. 33 % DNA-like and 67 % ribosomal RNA-like material. Alternatively, the 14-S component might be a homogeneous molecule with a composition intermediate between that of the DNA and the ribosomal RNA in each species. In a following paper³² it has been observed that the base composition of the newly formed RNA after 10–15 sec ³²P incorporation into growing bacterial cells is still very like the base composition measured after as long as 4 min incorporation, in each of the five species. As this material is equivalent to the 14-S material in the cells, the proportions of DNA-like and ribosomal RNA-like structures in the component shown above probably also exist at these very brief incorporation periods.

It must be emphasized that the base compositions of the 14-S components of bacterial cells given above represent only apparent compositions as probably the pool of material in the 14-S component has not been saturated with ³²P at the times above. An absolute base composition of this RNA can only be obtained by isolation of the fraction at incorporation times known to be adequate to saturate its pool. Further experiments to this end will be reported in a subsequent paper³².

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III.B.5 The Synthesis and Kinetic Behavior of Deoxyribonucleic Acid-Like Ribonucleic Acid in Bacteria

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SUMMARY

A study has been made of RNA synthesis and the changes in nucleotide composition of newly formed RNA in five species of bacteria. The kinetics of incorporation of [¹⁴C]uracil and [³²P]orthophosphate have also been studied.

1. The composition of the RNA synthesized after very short exposures to ³²P is intermediate between that of the bacterial DNA and ribosomal RNA.

2. The rapidly labeled 14-S RNA fraction is heterogeneous and can be separated into 30-40 % DNA-like RNA (D-RNA) and the rest (60-70 %) ribosomal-like RNA (R-RNA).

3. The changing base composition of newly formed RNA as a function of time is mainly the result of dilution of the 14-S material with an increasing proportion of ribosomal RNA.

4. Kinetic studies indicate that the degradation of D-RNA provides material used in the synthesis of soluble RNA and DNA.

5. In chloramphenicol, D-RNA is labeled at the normal rate, but the label is not so rapidly lost by degradation.

6. These experiments lead to an estimate of about 1 % for the amount of D-RNA in exponentially growing bacteria, and approx. 2-3 min for its average lifetime.

7. The number of D-RNA molecules thus estimated is one-tenth the number of ribosomes, assuming an average molecular weight of 200 000. If all the D-RNA acts as template for protein synthesis, then for a coding ratio of 3 nucleotides per amino acid the average rate of peptide-bond synthesis is about 0.4/sec/coding unit and each triplet could direct the incorporation of 75 amino acids during its average lifetime of 2.5 min.

INTRODUCTION

The existence in bacteria of a fraction of RNA with a DNA-like base composition first became apparent from the studies of phage-infected cells¹. More recently, other

Abbreviations: C, cytidylic acid; A, adenylic acid; G, guanylic acid; U, uridylic acid; T, thymidylic acid; s-RNA, soluble RNA; R-RNA, an RNA having the base composition of ribosomal (70-S) RNA of bacteria; D-RNA, an RNA having a composition like the DNA of the bacteria.

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studies have been directed towards the detection of such an RNA fraction in uninfected growing bacteria and yeast. While considerable success has been achieved in the detection of a rapidly labeled fraction of RNA²⁻⁴ the metabolic role of this RNA has not been established. The DNA-dependent synthesis of complementary RNA has, however, been successfully studied in systems *in vitro*⁵⁻⁸.

Published studies of rapidly labeled RNA contain two conflicting sets of observations. On the one hand it is evident from kinetic studies of the flow of [³²P]-orthophosphate or [¹⁴C]uracil in and out of this fraction⁹ that it can be considered predominantly, at least, as a precursor to the ribosomal RNA. On the other hand, analyses of the pulse-labeled total RNA^{10,11} and the purified 14-S fraction¹² indicate an apparent base composition intermediate between that of ribosomal RNA and the bacterial DNA. Consequently, depending on the type of observations made, the rapidly labeled fraction has been described as mostly ribosome precursor or mostly the "messenger" or informational RNA postulated to be necessary for the genetically directed synthesis of specific proteins on ribosomes¹³⁻¹⁵. KITAZUME, YCAS AND VINCENT¹⁶ have attempted to resolve this paradox by concluding that in yeast the DNA-like RNA fraction is an obligatory precursor in the formation of ribosomal RNA.

It therefore seemed possible that the 14-S RNA fraction contained molecules of different compositions and functions. The present study was undertaken to correlate kinetic and composition measurements and to determine the rate of synthesis of D-RNA. The term DNA-like RNA or D-RNA has been used when the polynucleotide composition is observed. Template RNA designates RNA observed to act as template for protein synthesis and messenger RNA describes RNA shown to have all the properties postulated by JACOB AND MONOD¹³.

METHODS

The five species of bacteria employed have been previously described¹². All experiments were carried out using cultures growing exponentially at 37° in aerated Tris-glucose media containing 8 mg/l of phosphorus. Under such conditions the approximate mean generation times were: *Aerobacter aerogenes*, 42 min; *Escherichia coli* and *Bacillus subtilis*, 50 min; *Proteus vulgaris*, 90-120 min; *Pseudomonas aeruginosa*, 120 min.

Techniques of labeling with [³²P] and [¹⁴C]uracil, preparation and analysis of cell extracts by sedimentation, and simultaneous counting of ¹⁴C and ³²P radioactivity have been described⁹.

Nucleotide analyses were made by the method described by MIDGLEY¹².

RNA was prepared by the addition of sodium dodecyl sulphate to cells broken in the French pressure cell followed by phenol extraction^{17,18}.

In analyses by chromatography on the methylated beef albumin-kieselguhr columns of MANDELL AND HERSHEY¹⁹, unlabeled cells were added to the pulse-labeled samples before disruption to facilitate quantitative extraction of RNA. After phenol extraction the RNA was precipitated with 3 vol. of cold 95 % ethanol. 3-5 mg of RNA were adsorbed to the column and eluted with a linear gradient of NaCl from 0.4-1.1 M in 300 ml of 0.02 M phosphate buffer (pH 6.7).

Chloramphenicol, a Parke-Davis product, was used at a concentration of 200 mg/l (see ref. 20).

RESULTS

I. Nucleotide composition of pulse-labeled RNA

The nucleotide composition of labeled RNA formed after various times of exposure to ^{32}P has been measured for each of the five species of bacteria studied. In each case ^{32}P was added to the bacteria growing exponentially in the low phosphorus-Tris medium. Growth and incorporation were terminated in samples taken at intervals by adding to an equal volume of 10 % trichloroacetic acid. Since the method of nucleotide analysis itself¹² represented an adequate purification of the 2',3'-nucleotides, no attempt was made to remove other macromolecules from the RNA. Likewise DNA contamination is of no account since DNA is unaffected by the alkaline hydrolysis conditions. On the other hand labeled 5'-nucleotides from the trichloroacetic acid-soluble pool are serious contaminants. For all but very brief exposures to ^{32}P , it was found that 5'-nucleotide contamination could be eliminated by filtration of the trichloroacetic acid precipitate through membrane filters followed by multiple washes with 5 % trichloroacetic acid²¹. After washing, the filters were dried and kept frozen until hydrolyzed. In this way nucleotide compositions of the pulse-labeled portion of the total RNA could be measured without the possibility of fractionation or selective degradation during purification steps.

For samples taken after exposures to ^{32}P for a period shorter than 1 % of the generation time this technique proved insufficient. In this time range the specific radioactivity of the 5'-nucleotides may be 1000 times that of the 2',3'-nucleotides (see Fig. 1) and small amounts of soluble material can seriously contaminate the nucleotide peaks in the analysis. Therefore very early samples were chilled rapidly by addition to crushed, frozen medium and washed in cold Tris-magnesium buffer. The frozen cells were disrupted in the French pressure cell and the effluent was added directly to phenol at 37°. The RNA was then purified by means of a second phenol extraction and two alcohol precipitations. The final alcohol precipitate was taken up in buffer and made up to 5 % trichloroacetic acid. Filtration and washing on the filter completed the purification. In this way samples of labeled RNA could be analyzed from cells given ^{32}P for periods as short as 10 sec.

Tables I-V contain the results of these nucleotide analyses of RNA after ^{32}P exposures of 10 sec to several hours. The tables also compare the G+C/A+T ratio

TABLE I
NUCLEOTIDE COMPOSITION OF NEWLY FORMED RNA IN *Proteus vulgaris* (MOLE %)

Time of labeling with isotope (min)	*	C	A	G	U(T)	$\frac{G+C}{A+U(T)}$
0.25	0.0019	23.5	26.4	26.6	23.5	1.00
1	0.0076	23.5	27.3	26.4	22.8	1.00
2	0.015	23.5	27.1	26.7	22.7	1.01
4	0.030	22.7	26.7	28.1	22.5	1.03
8	0.061	22.9	26.3	28.7	22.1	1.07
14	0.106	23.0	25.6	30.6	20.8	1.16
20	0.152	22.5	24.9	31.6	21.0	1.18
40	0.304	22.4	24.8	31.5	21.3	1.17
360	2.72	22.6	24.6	32.0	20.8	1.21
DNA		19	31	19	31	0.61

* Fraction of time e (see text).

TABLE II
NUCLEOTIDE COMPOSITION OF NEWLY FORMED RNA in *Bacillus subtilis* (MOLE %)

Time of labeling with isotope (min)	*	C	A	G	U(T)	$\frac{G+C}{A+U(T)}$
0.17	0.0023	23.5	25.5	27.3	23.7	1.03
0.5	0.0069	24.0	25.1	27.9	23.0	1.08
1	0.014	22.8	25.5	27.2	24.5	1.00
2	0.028	23.3	25.6	27.7	23.4	1.04
4	0.055	23.7	26.2	28.0	22.1	1.07
8	0.100	22.5	26.2	29.3	22.0	1.08
14	0.193	22.1	25.5	31.5	20.9	1.15
28	0.386	21.6	26.0	31.7	20.7	1.14
360	4.96	22.1	25.5	31.4	21.0	1.15
DNA		21	29	21	29	0.72

* Fraction of time e (see text).

TABLE III
NUCLEOTIDE COMPOSITION OF NEWLY FORMED RNA in *Escherichia coli* (MOLE %)

Time of labeling with isotope (min)	*	C	A	G	U(T)	$\frac{G+C}{A+U(T)}$
0.17	0.0023	22.8	25.1	28.8	23.3	1.11
0.5	0.0069	23.2	25.3	29.8	21.7	1.13
1	0.014	23.4	24.8	30.1	21.7	1.14
2	0.028	22.9	25.0	29.5	22.6	1.10
5	0.069	22.4	26.0	29.9	21.7	1.10
10	0.138	22.0	25.1	30.9	22.0	1.12
20	0.276	20.9	25.6	32.5	21.0	1.14
30	0.414	21.4	25.8	33.3	19.5	1.20
50	0.690	21.6	25.5	32.8	20.1	1.18
360	4.96	21.9	25.1	32.6	20.4	1.18
DNA		26	24	26	24	1.08

* Fraction of time e (see text).

TABLE IV
NUCLEOTIDE COMPOSITION OF NEWLY FORMED RNA in *Aerobacter aerogenes* (MOLE %)

Time of labeling with isotope (min)	*	C	A	G	U(T)	$\frac{G+C}{A+U(T)}$
1	0.012	23.5	23.4	32.0**	21.1	1.25
2	0.023	24.1	24.5	30.2	21.2	1.19
4	0.046	23.4	24.8	30.3	21.5	1.17
8	0.092	22.8	24.9	30.7	21.6	1.17
14	0.161	22.1	25.4	31.4	21.1	1.15
20	0.230	21.9	25.7	31.2	21.2	1.16
40	0.460	21.9	25.7	31.5	20.9	1.13
360	4.14	22.0	25.6	31.7	20.7	1.16
DNA		28	22	28	22	1.27

* Fraction of time e (see text).

** The measurement of G in the sample labeled for 1 min is inaccurate due to contamination by orthophosphate.

TABLE V

NUCLEOTIDE COMPOSITION OF NEWLY FORMED RNA IN *Pseudomonas aeruginosa* (MOLE %)

Time of labeling with isotope (min)	*	C	A	G	U(T)	$\frac{G+C}{A+U(T)}$
0.25	0.0015	26.2	21.4	31.9	20.5	1.39
2	0.012	25.3	21.6	32.3	20.8	1.36
4	0.025	25.4	21.1	31.9	21.6	1.34
8	0.050	25.8	20.8	31.6	21.8	1.35
14	0.086	26.1	21.8	31.0	21.1	1.33
20	0.126	24.7	22.0	31.7	21.6	1.29
40	0.252	23.9	23.5	32.0	20.6	1.27
60	0.388	22.6	25.5	31.2	20.7	1.16
360	2.07	22.2	25.7	31.3	20.8	1.15
DNA		32	18	32	18	1.78

* Fraction of time e (see text).

of the DNA (which varies from 0.61 to 1.75 among the five species^{22,23}) with the $G+C/A+U$ ratio of the newly formed RNA. Assuming that the newly formed RNA is a mixture of two types (see Section 3), comparison of the $G+C/A+U$ ratio with that for the bacterial DNA and the ribosomal RNA ($G+C/A+U = 1.15$) or the total RNA ($G+C/A+U = 1.20$)¹² gives a measure of the amount of labeled RNA in the sample having a nucleotide composition resembling DNA. Such a comparison is, of course, most useful in cases where the composition of the DNA and the ribosomal RNA are very different, *i.e.* *P. vulgaris*, *B. subtilis* and *Ps. aeruginosa*. Even where these differences are small, *i.e.* *E. coli* and *Aerobacter aerogenes*, the rapidly labeled RNA and the total RNA are not identical.

Two features are immediately clear from examination of the data in Tables I–V. In each bacterial species, the composition of the RNA at the earliest times is that which would result from a mixture of 30–40 % having a DNA-like composition and 60–70 % ribosomal¹². Moreover the change in base composition or $G+C/A+U$ ratio is not an especially rapid one. In general there is little difference among the first three or four analyses representing times from about 0.3 % to about 4 % of a generation time. If the nucleotide composition represents the weighted mean between different amounts of two different types of RNA molecules, the relative amounts of radioactivity in the two different molecules present does not change during the first 5 % or so of the generation time. There is no indication of an extremely rapidly labeled component. Analyses made during the period between 5 % and 20 % of a generation time show a change in composition towards that of ribosomal RNA. At times greater than 20 % of a generation time, the nucleotide composition of the labeled fraction is indistinguishable from that of the total RNA.

2. Fractionation of pulse-labeled RNA

The early labeled RNA can be separated into two fractions of different composition by differential dissociation from ribosomes. The pulse-labeled cells of the four bacterial species shown in Table VI were prepared by exposure for 3 min to ³²P. After chilling and washing, the cells were broken in Tris buffer containing 10^{-2} M MgCl₂. Following removal of cell debris by a short centrifugation (2 min at $105\,000 \times g$)

TABLE VI
FRACTIONATION OF 3 MIN ^{32}P PULSE-LABELED RNA BY WATER TREATMENT

G+C/A+T in DNA	<i>Ps. aeruginosa</i>		<i>A. aerogenes</i>		<i>E. coli</i>		<i>P. vulgaris</i>	
	1.75		1.27		1.08		0.61	
	Supernatant	Precipitate	Supernatant	Precipitate	Supernatant	Precipitate	Supernatant	Precipitate
C	28.4	22.0	—	21.9	25.2	21.8	—	22.1
A	20.8	25.5	—	25.6	24.4	25.0	—	25.5
G	31.2	31.6	—	31.6	26.9	32.6	—	31.8
U	19.6	20.9	—	20.9	23.5	20.6	—	20.6
$\frac{\text{G+C}}{\text{A+U}}$	1.47	1.15		1.14	1.09	1.19		1.17
Per cent ^{32}P	52	40	5	46	30	70	12	34
Per cent [^{32}P] RNA recovered	92		51		100		46	

most of the ribosomal material and about 90 % of the labeled RNA were pelleted by means of a 45-min centrifugation at 40 000 rev./min. The ribosome pellet (2–5 mg) and the interior of the centrifuge tube were rinsed with distilled water at 2° to remove all traces of the buffer. The pellet was then resuspended in distilled water and centrifuged at 40 000 rev./min for 120 min. The magnesium concentration resulting largely from the bound ribosomal ions²⁴ can be estimated at $2\text{--}4 \cdot 10^{-4} M$.

The top half of the supernatant was removed and trichloroacetic acid precipitated and filtered. The pellet was also resuspended, precipitated and filtered. Table VI contains the results of the nucleotide analyses of the various fractions together with the fraction of labeled macromolecules recovered. In the case of *E. coli* the results are the mean of six different experiments, while the other results are the mean of two experiments.

The separation technique is most successful for *E. coli* material. Very little of the pulse-labeled RNA was degraded during the procedure and the fractions obtained had nucleotide compositions very close to those of pure ribosomal RNA and of *E. coli* DNA. Distortions of the real nucleotide composition by a combination of unequal labeling of pool nucleotides⁹ and nonrandomness in DNA sequences^{25–28} are not apparent in the measured composition of the D-RNA. Apparently the “water-shock” treatment causes the DNA-like fraction to become disassociated from the ribosomes leaving behind the labeled RNA which resembles ribosomal RNA. Examination of water-treated 70-S ribosomes in the analytical ultracentrifuge shows that the ribosomes have been dissociated into two fractions of approx. 60-S and 10-S (see ref. 29) unlike the usual dissociation into 50-S and 30-S ribosomes. The supernatant fraction from both *E. coli* and *Ps. aeruginosa* appears to be quite similar to the DNA in composition.

Unfortunately the treatment results in the degradation of a large proportion of the pulse-labeled RNA in both *P. vulgaris* and *A. aerogenes* presumably due to RNAase liberation. Even there, however, it is evident that the fraction remaining with the ribosomes is purely ribosomal in base composition. The degradation, therefore, appears to be selective, the DNA-like fraction being preferentially destroyed. Again the rapidly labeled fractions appear to contain two types of molecule. Sucrose density-gradient sedimentation of both “water-shock” supernatant and pelleted

labeled RNA in the presence of 10^{-4} M Mg^{++} showed similar broad peaks of radioactivity with a 14-16-S maximum.

3. Kinetic studies of RNA synthesis with ^{32}P

The delay in incorporation of ^{32}P into RNA brought about by the large pool of trichloroacetic acid-soluble RNA precursors prevents direct correlation of base composition changes (Tables I-V) with published studies of the flow of [^{14}C]uracil into ribosomes⁹. In order to make such a comparison four samples of *P. vulgaris* extracts were prepared after various exposures to ^{32}P . Sedimentation analysis on sucrose gradients allowed measurement of the fraction of labeled RNA present in the various species of ribosomes and precursors. In addition the rate of uptake of ^{32}P into the total RNA gave a measure of the trichloroacetic acid-soluble pool.

Fig. 1 shows the incorporation curve of ^{32}P into *P. vulgaris*. The data were obtained from samples of whole cells and trichloroacetic acid-extracted cells filtered through membrane filters²¹. In addition some samples at late times were extracted

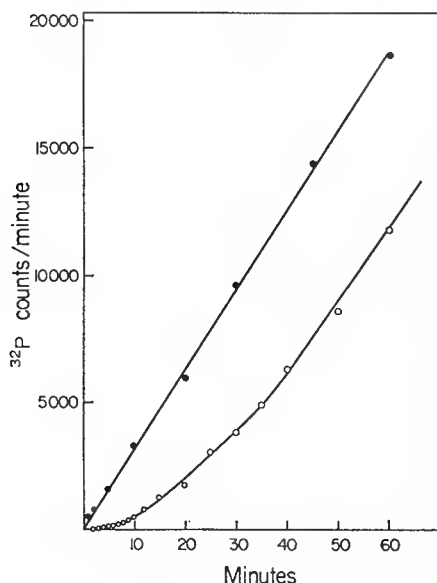


Fig. 1. The incorporation of ^{32}P as orthophosphate into exponentially growing *Proteus vulgaris*. ●-●, Total cellular ^{32}P ; ○-○, trichloroacetic acid-precipitable ^{32}P .

with hot ethanol in order to make corrections for the lipid phosphorus. An estimate of the size of the phosphorus pool was made by the method of kinetic analysis already described³⁰. Times were converted to τ where $Q = Q_0 e^{\tau}$ gives the growth of the cells or any component³⁰. Normalization of the data so that the labeled fraction of nucleotide phosphorus fitted $1 - e^{-\tau}$ (see ref. 9) gave the top curve in Fig. 2. Similar treatment of the data for uptake of ^{32}P into total RNA gave the second curve from which one may obtain the size of the phosphorus pool. The phosphorus pool is, of course, complex containing mono-, di-, and tri-phosphates of the various nucleosides, and inorganic phosphate. Moreover there are a great number of reactions among

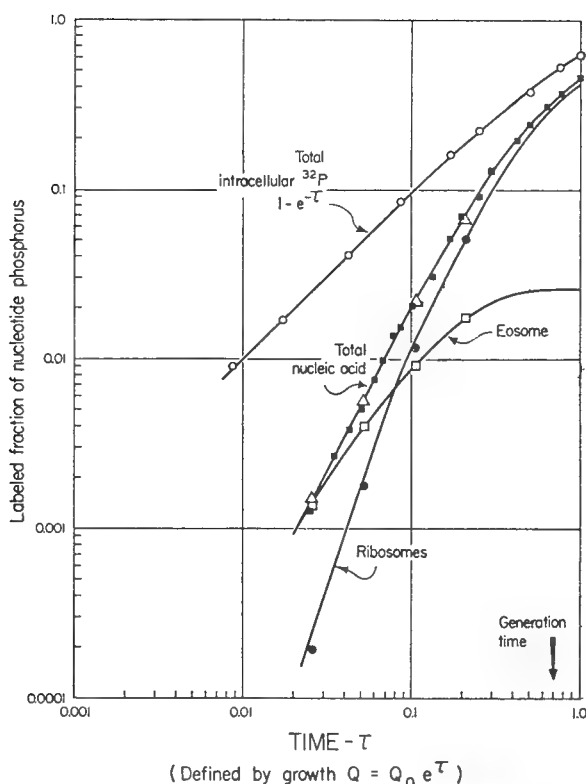
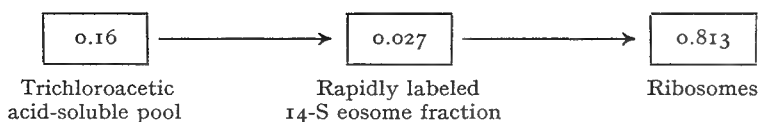


Fig. 2. Log-log. plot of the time course of the labeling of the RNA of *Proteus vulgaris*. ○—○, labeled fraction of nucleotide phosphorus for the total cell and ■—■, total nucleic acid (data from Fig. 1). △—△, total nucleic acid; □—□, 14-S eosome fraction and ●—●, total ribosomal material (data from Fig. 3). The lines drawn were calculated from the equations²⁸ for the case where the phosphorus precursor pool is 16 % of the total nucleotide phosphorus and the 14-S fraction is 2.7 % of the total RNA (see text).

the various components and a portion of the phosphorus is destined for lipid synthesis. Nevertheless it behaves for kinetic purposes as a single delay pool. The size of the pool thus measured is the ratio of the phosphorus in these small molecules to the total phosphorus in nucleic acid and the precursor molecules. The above procedure gave 0.16 for the size of the phosphorus precursor pool in *P. vulgaris*. (Total phosphorus in nucleic acid and precursors = 1.) The curve drawn through the points in Fig. 2 was calculated using this value³⁰.

Four samples of *P. vulgaris* extracts after growth for two generations in [¹⁴C]-uracil and 4-, 8-, 16- and 32-min exposure to ³²P. The extracts were fractionated by sedimentation and the fractions assayed for trichloroacetic acid-precipitable ¹⁴C and ³²P (Fig. 3). Such pulse-labeling experiments are the inverse of those already described in detail in which ³²P was used as the steady label and ¹⁴C as the pulse label⁸. The labeled fraction of the nucleotide phosphorus was computed for the total RNA, the rapidly labeled 14-S fraction and the bulk 30-S and 50-S ribosomes, and plotted on Fig. 2. The points for the total RNA fit the total incorporation curve already described. The other two sets of points fit well to theoretical curves calculated for the model below according to Eqn. 7 of BRITTEN AND MCCARTHY³⁰.



The quantity of the rapidly labeled fraction has been assumed to be equal to 2.7 %, the best estimate from experiments with *E. coli*⁹. It should be pointed out, however, that the curves are relatively insensitive to the magnitude of this number except at late times.

It is apparent then that the kinetics of ³²P labeling of the rapidly labeled fraction are consistent with a precursor-product relationship between the 14-S fraction and ribosomes. These kinetic studies with a different organism and a different labeling scheme are in agreement with the *E. coli* studies already published, after the inclusion of the 16 % trichloroacetic acid-soluble pool.

4. Correlation of nucleotide compositions with kinetic data

The sedimentation analyses of Fig. 3 give a measure of the changing distribution of ³²P among the various RNA-containing components of the cell. It is therefore

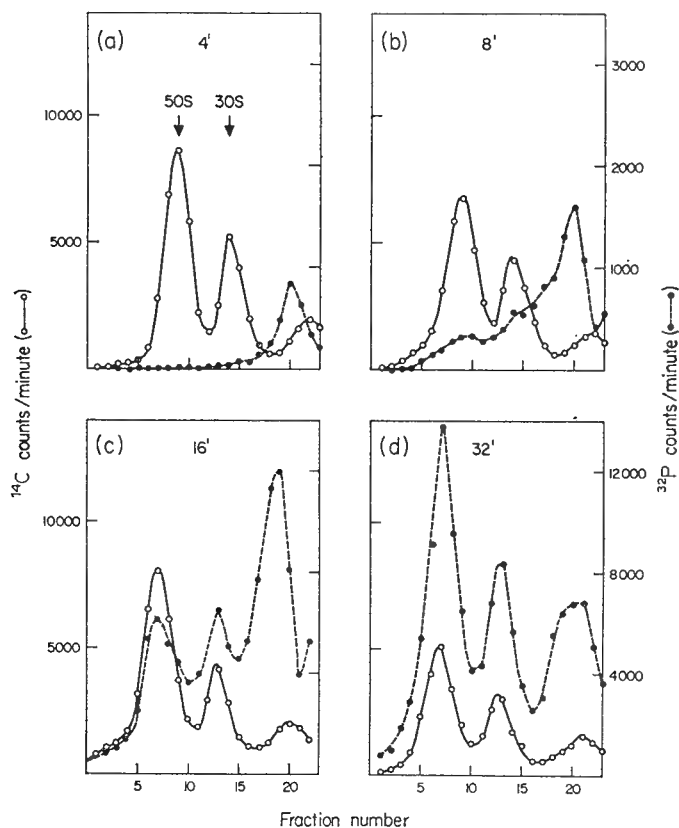


Fig. 3. Sedimentation analysis of four total cell extracts from *Proteus vulgaris* labeled for three generations with [¹⁴C]uracil and given (a) a 4-min exposure to [³²P]orthophosphate (b) 8 min, (c) 16 min, (d) 32 min. Extracts prepared from about 0.5 mg dry wt. of cells in Tris-HCl, 0.01 M (pH 7.4); MgCl₂, 10⁻⁴ M. Centrifugation 150 min at 37 000 rev./min at 4°.

possible to re-examine the measurements of the base composition of the total RNA in terms of the relative amounts of ribosomes and 14-S component present. A juxtaposition of the data of Table I and Fig. 3 is shown in Fig. 4.

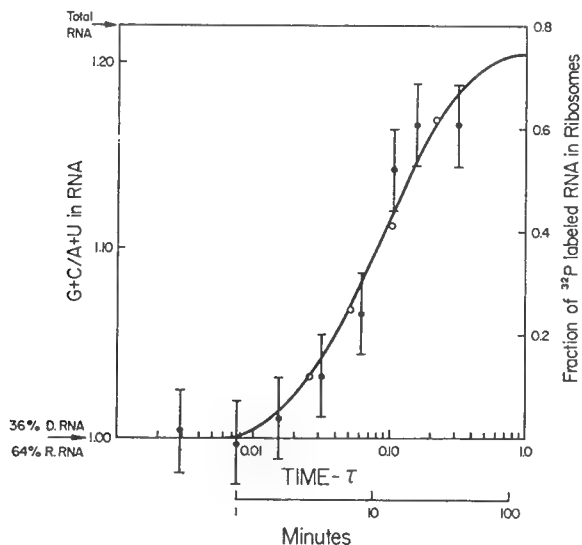


Fig. 4. A juxtaposition of the data of Table I and Fig. 3. The nucleotide analyses (I-I) are plotted against time in terms of $G+C/A+U$ ratios. The left-hand scale runs from 1.00, the average of the earliest determinations, to 1.22, the value for total RNA. The fraction of the total ^{32}P label present in ribosomes (30 S or greater) (○-○) has been plotted on a scale from zero to 0.8. The data are those shown in Fig. 3. The line drawn through the points is a theoretical one for the fraction of ^{32}P -labeled RNA in ribosomes as a function of time calculated as described in the text.

The time course of the $G+C/A+U$ ratio is plotted on a scale which runs from 1.00 (the average of the earliest determinations) to 1.22, the value for total RNA of *P. vulgaris* after long periods of labeling (Table I). Since the value for DNA or pure D-RNA on this scale is 0.61 (average of the results of LEE *et al.*²² and SPIRIN *et al.*²³) the origin, representing the zero-time composition, would correspond to 36 % D-RNA and 64 % R-RNA. The fraction of the total ^{32}P -labeled RNA present in ribosomes, *i.e.* all counts sedimenting at 30 S or greater, was computed for each time point in Fig. 3. These are plotted in Fig. 4 on a scale running from zero to 0.8 since the remaining 20 % of the RNA is s-RNA. Finally the expected function for the change of the fraction of the label present in ribosomes was calculated from the theoretical curves already plotted in Fig. 3.

At early times while all the ^{32}P radioactivity is present in the 14-S fraction, the nucleotide composition remains essentially constant. Changes in composition towards that of the total RNA begin at the time when label first enters ribosomes. Thus the change in $G+C/A+U$ can be accounted for by the increasing relative proportion of labeled ribosomes of pure RNA composition. The data imply a rapidly labeled RNA fraction of 2-3 % of the total RNA with a time constant of 2-3 min, 1/3 of which is D-RNA. Since the composition of the 14-S fraction is constant, the lifetime of the two RNA molecules present must be very similar.

5. Composition of the isolated 14-S fraction

It is clear from Table I and Fig. 4 that the 14-S fraction shows no measurable change in $G+C/A+U$ ratio during its labeling. More direct evidence for the constancy of the composition of the 14-S fraction is shown in Table VII. At longer times when the total composition is beginning to change (Table I) and the 14-S fraction no longer contains all the radioactivity, very similar results were obtained indicating proportions

TABLE VII
NUCLEOTIDE COMPOSITION OF PURIFIED 14-S FRACTION OF *Proteus vulgaris* (MOLE %)

Time of labeling with isotope (min)	*	C	A	G	U(T)	$\frac{G+C}{A+U(T)}$
2	0.0148	23.5	27.1	27.1	23.3	1.02
5	0.0370	22.7	26.7	27.6	23.0	1.01

* Fraction of time e (see text).

of D-RNA and R-RNA of 1:2. The agreement between these late-time values of the purified 14-S fraction and the early-time total compositions indicates that unequal 5'-nucleotide-specific radioactivities did not seriously influence the early determinations¹². In the case of *P. vulgaris* base compositions of this fraction prove to be constant throughout the range from 15 sec to 5 min.

6. Resolution of pulse-labeled RNA by chromatography

The technique of chromatography on columns of methylated beef-serum albumin adsorbed on kieselguhr, has been used to study the pulse labeling of various RNA fractions. The fractionation of DNA by this column has been shown by MANDELL AND HERSHEY¹⁹ to depend on the molecular weight of the nucleic acid. Furthermore, preparations of total bacterial nucleic acid prepared by the phenol method are resolved into s-RNA, DNA and two peaks of ribosomal RNA resulting from the two sizes of molecules (16 S and 23 S) found after phenol treatment³¹. This technique has been employed in the fractionation of the rapidly labeled RNA and in following the uptake of [¹⁴C]uracil and [³²P]orthophosphate into the various nucleic acid fractions.

RNA labeled after brief exposure to the isotopes appears in three peaks other than s-RNA and DNA, none of which is exactly coincident with the two peaks of ribosomal RNA^{12,31} (Figs. 5 and 7). This distribution does not, however, reflect a separation into RNA's of different nucleotide composition. The relative proportions of label among the three peaks is entirely reproducible provided that the conditions of phenol extraction are such that they preserve the normal proportions of the two sizes of ribosomal RNA. If the RNA is extracted in the presence of low concentrations of magnesium some of the 23-S RNA may be converted to 16-S RNA³². Such treatment also changes the distribution of the early labeled RNA among the three peaks. Fig. 5 shows such an elution diagram of ³²P pulse-labeled RNA of *P. vulgaris*. There is still no fractionation into materials of different nucleotide composition (Table VIII). Thus while it appears that the three peaks of radioactivity may be aggregates

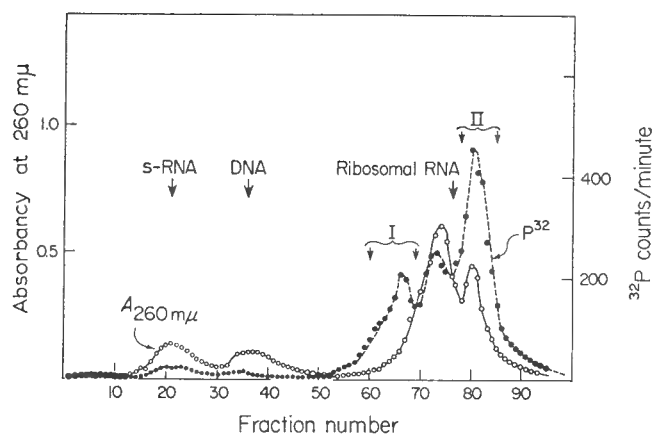


Fig. 5. Elution diagram from a column of methylated beef albumin on kieselguhr of a sample of RNA from *Proteus vulgaris* labeled for 90 sec with ^{32}P . RNA extracted from cells broken in Tris-HCl, 0.01 *M* (pH 7.4) containing 10^{-4} *M* MgCl_2 . Elution with 300 ml of 0.02 *M* phosphate buffer (pH 6.7) containing a linear gradient of sodium chloride from 0.4 to 1.1 *M* NaCl.

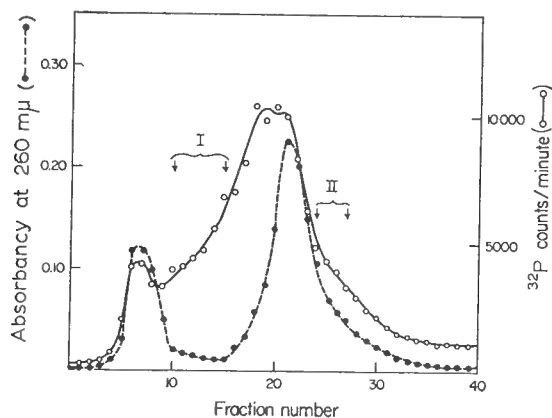


Fig. 6. Elution diagram from a column of methylated beef albumin on kieselguhr of a cell extract in Tris-HCl, 0.01 *M* (pH 7.4), 10^{-4} *M* MgCl_2 , prepared from *B. subtilis* cells given a 3-min exposure to $[^{32}\text{P}]$ orthophosphate. Elution with 200 ml of Tris-HCl, 0.01 *M* (pH 7.4) containing 10^{-4} *M* MgCl_2 and a linear gradient of sodium chloride from 0.5 to 1.0 *M*.

TABLE VIII

NUCLEOTIDE COMPOSITIONS OF TWO FRACTIONS OF *Proteus vulgaris* 90 SEC ^{32}P PULSE-LABELED RNA RESOLVED ON A METHYLATED BEEF-ALBUMIN-KIESELGUHR COLUMN (FIG. 5).

	C	A	G	U(T)
Total ^{32}P -labeled RNA	23.5	26.9	26.7	22.9
Peak I	23.5	25.7	27.2	23.6
Peak II	24.7	25.5	27.0	22.8

of the pulse-labeled RNA with the 16-S and 23-S material, or with itself, partition of the D-RNA and the R-RNA among them is closely similar.

The appearance of essentially all of the early labeled RNA in this region makes

it very convenient to compare the kinetics of labeling of this mixture of D-RNA and R-RNA with that of the s-RNA and DNA. Two series of samples of *E. coli* RNA were chromatographed in this way, one prepared from cells which had been pulse labeled with [¹⁴C]uracil and the other from ³²P pulse-labeled cells.

Fig. 7 shows two of the eight analyses made of RNA prepared from cells labeled with ³²P for 3 generations, and for periods of up to 15 min with [¹⁴C]uracil. Fig. 7(a)

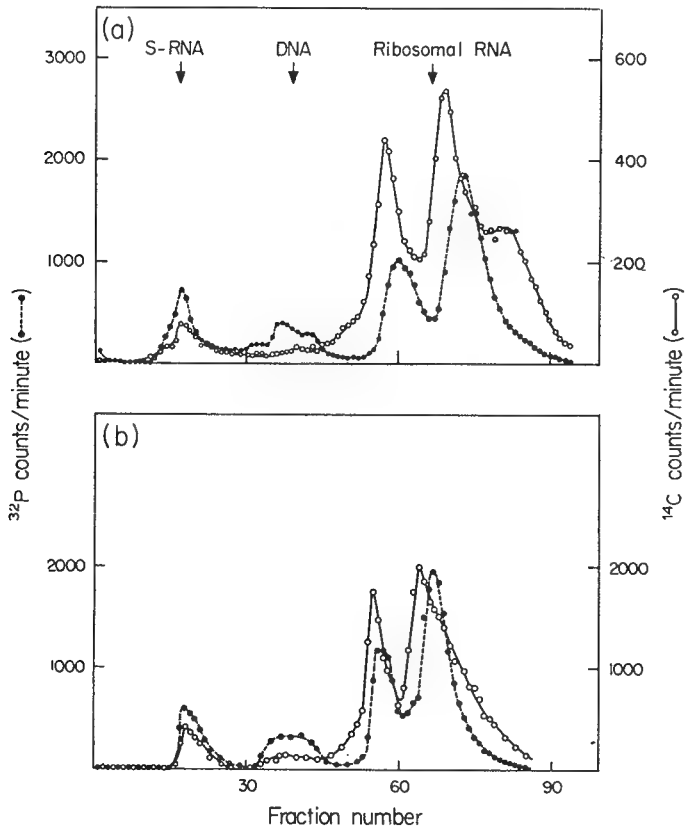


Fig. 7. Elution diagram from columns of methylated beef albumin on kieselguhr of two samples of RNA from *E. coli* grown for three generations in [³²P]orthophosphate and given (a) 1 min, (b) 3.25 min [¹⁴C] uracil. Elution as in Fig. 5.

(1 min [¹⁴C]uracil incorporation) shows the characteristic three peaks of pulse-labeled material in the high-molecular-weight region not coincident with the two peaks of ³²P-labeled RNA. The specific radioactivity, in this case given by the ratio of ¹⁴C counts/minute to ³²P counts/minute, is about three times higher in the high-molecular-weight region, taken as a whole, than in the s-RNA^{15,30}. By a labeling time of 3.25 min, the time of the second analysis shown in Fig. 7(b), the difference in specific radioactivity is not as noticeable. At even later times (Fig. 11(b)) most of the ¹⁴C radioactivity appears under the two main peaks of ³²P-labeled RNA.

Similar elution diagrams were obtained for the other five points after 40 sec and 2, 4.5, 7 and 10 min. The specific radioactivities of s-RNA and the mixture of

D-RNA and R-RNA were obtained by summation of the counts/min throughout the whole region. After making the appropriate correction⁹ the results were plotted in Fig. 8. The entry of uracil into s-RNA is subject to a delay of just over 1 min, not shown in the labeling of high-molecular-weight RNA. The labeling of DNA is delayed to a similar extent.

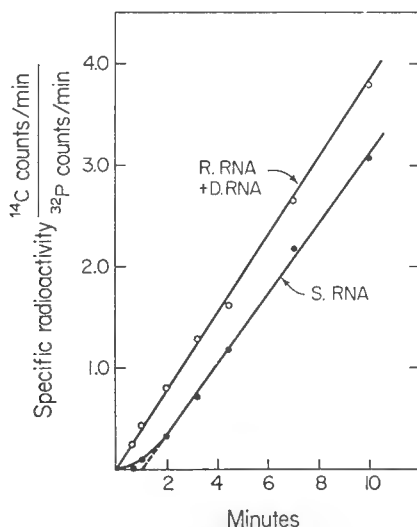


Fig. 8. Plot of the specific radioactivities of s-RNA (●—●), and the mixture of D-RNA and R-RNA (○—○) as ratios of ^{14}C counts/min to ^{32}P counts/min against time. Data from Fig. 7, and 5 other analyses.

The relative delay in s-RNA synthesis is not a special feature of uracil incorporation. When the identical experiment was performed using ^{32}P as the pulse label and $[^{14}\text{C}]$ uracil as the steady label very similar results were obtained. Fig. 9 shows an elution diagram of RNA labeled with ^{32}P for 5 min. The lower specific radioactivity, *i.e.* ratio of ^{32}P counts/min to ^{14}C counts/min and the separation of ^{32}P counts

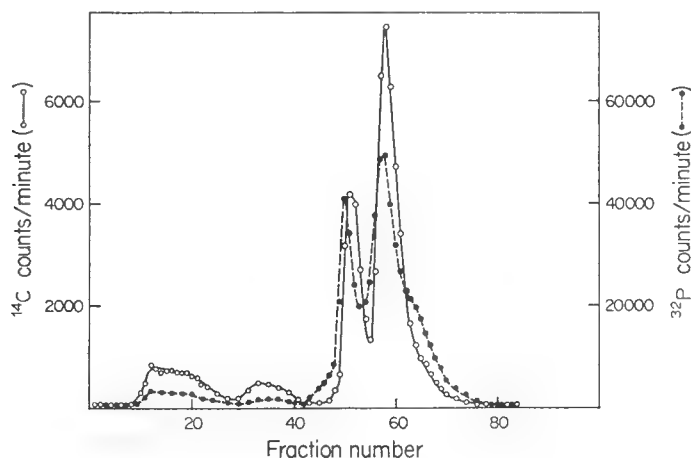


Fig. 9. Elution diagram from a column of methylated beef albumin on kieselguhr of a sample of *E. coli* RNA from cells labeled for three generations with $[^{14}\text{C}]$ uracil and for 5 min with ^{32}P .
○—○, ^{14}C counts/min; ●—●, ^{32}P counts/min.

from the steady ¹⁴C label, are immediately evident. Similar analyses were made after 1, 2, 3, 7 and 10 min. The specific radioactivities of s-RNA and the mixture of D-RNA and R-RNA are plotted in Fig. 10. In spite of the curvature brought about by the large pool of acid-soluble ³²P it is possible to see a delay in the entry of ³²P into s-RNA and DNA relative to that of R-RNA and D-RNA of about 2 min.

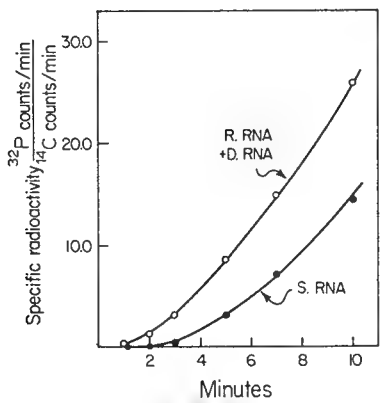


Fig. 10. The specific radioactivities of s-RNA (●—●) and the mixture of D-RNA and R-RNA (○—○) as a function of time plotted as ratios of ³²P counts/min to ¹⁴C counts/min. Data from Fig. 9 and 5 other analyses.

Thus the labeling of both the pyrimidine bases and the phosphorus atoms of newly synthesized s-RNA and DNA are delayed relative to other RNA by a pool of nucleotide material equivalent to 1- or 2-min worth of RNA. The conclusion will be drawn, after the presentation of results in Section 7, that this delay is a consequence of the degradation of a fraction of the high-molecular-weight RNA, identified with D-RNA.

TABLE IX

NUCLEOTIDE COMPOSITION OF TWO FRACTIONS OF *Bacillus subtilis* 3 MIN ³²P PULSE-LABELED RNA RESOLVED ON A METHYLATED BEEF-ALBUMIN-KIESELGUHR COLUMN (FIG. 6).

	C	A	G	U(T)
Total ³² P-labeled RNA	23.4	25.4	27.5	23.7
Peak I	23.7	25.0	27.2	24.1
Peak II	23.3	25.5	27.6	23.6

7. The effect of chloramphenicol on the synthesis of D-RNA

It has been known for a number of years that the overall nucleotide composition of RNA synthesized in the presence of chloramphenicol is similar to that of normal bacterial RNA³⁴. Fractionation of *E. coli* chloramphenicol RNA on columns of DEAE-cellulose gives two components, one having the nucleotide composition of s-RNA, and the other approximately that of ribosomal RNA³⁵. After 10 min exposure to [¹⁴C]uracil and chloramphenicol, analysis of the extracts on the sucrose gradients shows all of the radioactivity in either s-RNA or the 14-S precursor peak³⁶. It therefore appeared that chloramphenicol brought about an accumulation of the

14-S material here under investigation. Further experiments were therefore directed at determining the relative quantities and behaviors of the D-RNA and R-RNA moieties.

A detailed analysis of the rate of change of the nucleotide composition of the RNA was made in the presence of chloramphenicol. Chloramphenicol at 200 mg/l was added to exponentially growing cultures of *E. coli* and *P. vulgaris*, followed 5 min later by ^{32}P . Samples were taken for nucleotide analysis as described previously (Section I) and the results tabulated in Table X. The results should be compared to those for uninhibited cultures shown in Tables I and III.

TABLE X
COMPOSITION OF NEWLY FORMED RNA DURING INCUBATION WITH CHLORAMPHENICOL (200 mg/l)

Time (min)	C	A	G	U	$\frac{G+C}{A+U}$
<i>Escherichia coli</i>					
2	22.5	25.0	29.8	22.7	1.05
4	22.9	25.6	29.4	22.1	1.06
7	22.7	25.3	29.4	22.6	1.09
20	21.7	25.0	31.4	21.9	1.13
40	22.4	25.0	31.2	21.4	1.15
60	22.8	25.3	30.9	21.0	1.16
Total RNA	22.1	25.2	32.5	20.2	1.20
<i>Proteus vulgaris</i>					
5	22.6	26.0	28.0	23.4	1.02
10	21.5	25.4	31.2	21.9	1.11
20	21.9	25.2	30.7	22.2	1.11
40	21.6	26.0	30.8	21.6	1.10
60	21.4	26.2	30.9	21.5	1.10
Total RNA	22.6	24.6	32.0	20.8	1.20

The compositions at the earliest times compare well to those for control cultures and indicate the synthesis of about 1/3 D-RNA and 2/3 R-RNA. Although the composition changes towards that for total RNA in the early stages as in the control, the nucleotide composition at late times differs significantly from that of total RNA. In the case of *E. coli* the difference in $G+C/A+U$ between 1.15 or 1.16 and 1.20 is hardly significant (although it also appears in the data of HOROWITZ *et al.*³⁷), but in *P. vulgaris* one can be confident of the reality of the difference between 1.10 and 1.20. If these apparent compositions are taken at their face value the RNA formed after 1 h exposure to chloramphenicol would consist of some 10–20 % D-RNA. The studies of KURLAND AND MAALØE³³ show an abnormally high fraction of s-RNA in the RNA synthesized after long exposures to chloramphenicol. It is therefore probable that the data of Table X indicate an even higher fraction of D-RNA since more s-RNA tends to increase the overall $G+C/A+U$ ratio. Thus these observations suggest that D-RNA and R-RNA are synthesized in the normal proportions in chloramphenicol but that the breakdown of D-RNA is greatly reduced.

Further studies of the nature of RNA synthesis in chloramphenicol were made by means of chromatographic analysis of the RNA. Chloramphenicol at 200 mg/l was added to exponentially growing *E. coli* prelabeled for three generations with ^{32}P , followed 5 min later by ^{14}C uracil. Control cells received no chloramphenicol. Samples taken at 1, 2, 4 and 8 min were washed and broken in the usual way and the purified RNA analyzed on columns of methylated beef albumin as described (Section 6).

The two analyses made after 8-min exposures are shown in Fig. 11. By this time RNA from uninhibited cells show a high degree of coincidence between the ^{14}C and ^{32}P counts. On the other hand the RNA from the inhibited culture has a higher

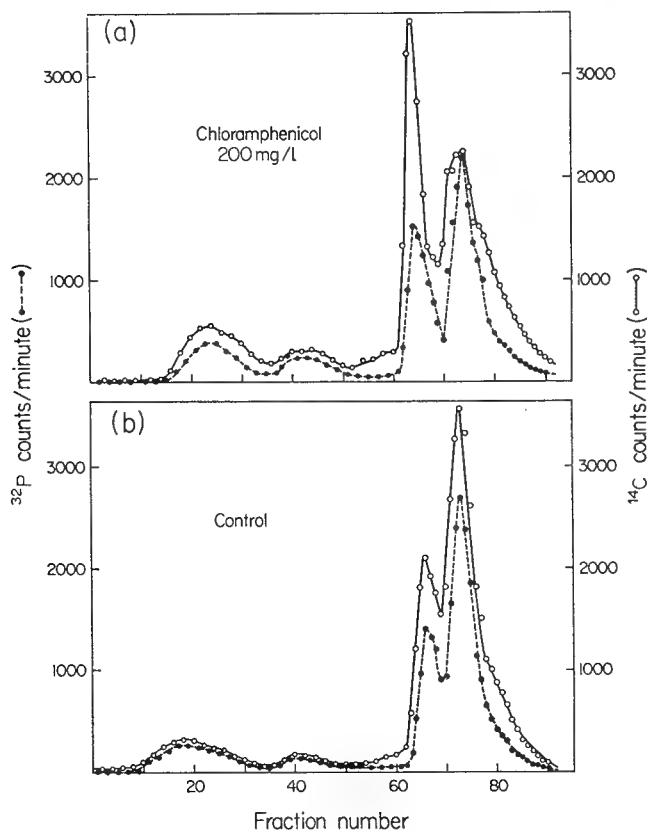


Fig. 11. Elution diagrams from columns of methylated beef albumin on kieselguhr of two samples of RNA from *E. coli* cells labeled for three generations with ^{32}P , and 8 min with ^{14}C uracil. (a) In the presence of 200 mg/l of chloramphenicol, (b) control.

proportion of the newly made RNA in the region of 16-S RNA. Moreover the remaining ^{14}C radioactivity appears in a peak much broader than that given by the 23-S ^{32}P -labeled RNA. In fact the specific radioactivity in this region is lowest at the peak, suggesting the existence of two overlapping components of ^{14}C radioactivity.

The analyses of chloramphenicol RNA at the earliest times, 1 and 2 min (not shown), are quite similar to those of the control except for the absence of the third

peak of ^{14}C radioactivity (see fig. 7(a)). At 4 min a degree of coincidence of the peaks of radioactivity lower than that in the control is already apparent.

Of greater interest is the plot of the specific radioactivities of s-RNA and D-RNA plus R-RNA shown in Fig. 12. It appears that the entry of $[^{14}\text{C}]$ uracil into s-RNA takes place without significant delay. A similar effect was apparent in the

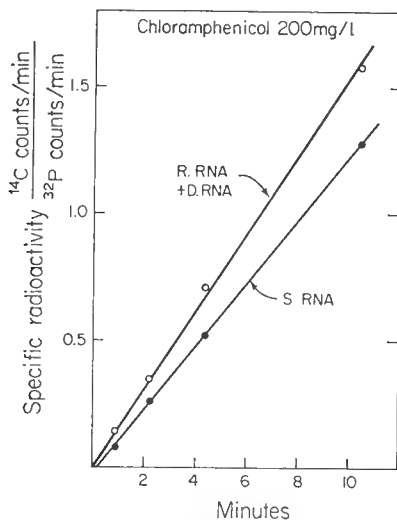


Fig. 12. The specific radioactivities of s-RNA (●—●), and the mixture of D-RNA and R-RNA (○—○) as a function of time from chloramphenicol-inhibited cultures plotted as ratios of ^{14}C counts/min to ^{32}P counts/min. Data from Fig. 11(a) and 3 other analyses.

labeling of DNA. The data from the control cultures gave the same 1-min delay as shown in Fig. 8. As the delay is abolished by chloramphenicol it does not appear to be the result of a special small-molecule precursor pool. On the other hand the lack of a delay may be correlated with the stability of D-RNA in chloramphenicol-inhibited cultures. Further discussion of this hypothesis will appear later.

DISCUSSION

A possible consequence of the messenger RNA hypothesis of JACOB AND MONOD¹³ is that these RNA molecules may survive long enough to specify the synthesis of only one protein molecule³⁸. Since these molecules would presumably be included in the D-RNA fraction, knowledge of its rate of synthesis is of major importance in considering the mechanism of protein synthesis.

There is, however, no evidence for an extremely rapid rate of turnover of the D-RNA fraction such that the rate of incorporation into these molecules is many times that into the stable RNA. In fact, nucleotide compositions measured at very early times are consistent with a flow of material into D-RNA equal to half that into stable ribosomal RNA. There is, of course, as in all isotope-labeling experiments, the possibility that the rate of uptake of label does not measure the true rate of synthesis. Thus in the present case, while it appears that the rate of RNA synthesis is 50 % higher than the flow into stable RNA, one may argue that D-RNA is being synthesized and degraded much more rapidly, in equilibrium with a chemically or

physically isolated pool. A similar argument must be applied to the incorporation of [^{14}C]uracil³⁹ and other bases⁴⁰. Since this would imply rigid separation between two or more pools of nucleotides and inaccessibility to labeling by either [^{14}C]uracil or ^{32}P , this process has not been considered further in the present discussion.

In all the five organisms studied the composition of the newly synthesized RNA is intermediate between that of D-RNA and R-RNA. Fractionation by water treatment of the 14-S component of *Ps. aeruginosa* and of *E. coli* into two RNA fractions provides a strong indication that it consists of a mixture of two types of molecule, one with a base composition like the DNA of the cell (D-RNA) and the other pure R-RNA. Although the fractionation was not as successful with RNA's of other bacteria, the results are consistent with the existence of two molecules with different compositions.

Detailed analyses of changes in nucleotide composition of the newly formed RNA indicate that the two types of molecule have very similar kinetics of incorporation. Thus the apparent composition of the 14-S RNA fraction remains constant and the changes in overall nucleotide composition appear to result from the degradation of the D-RNA moiety and the conversion of R-RNA to stable ribosomal material. Thus the compositional changes can be correlated with the appearance of radioactivity in stable ribosomal material which dilutes a 14-S component of constant composition¹². In effect, the lifetimes of D-RNA and R-RNA molecules in the 14-S fraction are closely similar, one being removed by degradation and the other by the addition of protein and conversion to ribosomes⁹.

The relative amounts of D-RNA and R-RNA synthesized in each of the five organisms studied appears to be the same. This ratio may have stoichiometric significance or merely be a function of the conditions of growth, since other authors report considerably higher fractions of D-RNA in pulse-labeled RNA. Bacteria undergoing a downward transition in growth rate¹⁵ and non-growing yeasts¹⁶ produce higher proportions of D-RNA. The G+C/A+U ratios for the DNA-like materials reported in the latter publication are, however, consistent with an equal mixture of D-RNA and R-RNA.

Chromatography of pulse-labeled RNA on methylated beef-albumin columns does separate the label from the bulk RNA but does not give any separation between D-RNA and R-RNA. Each of the three peaks obtained appears to consist of mixtures of the two components. The relative quantity appears to depend upon conditions of extraction. TAKAI *et al.*³¹ have shown that the sedimentation coefficients of the three peaks are approximately 12 S, 19 S and 26 S and suggested that they represent dimers and tetramers of a basic 12-S material. This would be in agreement with the suggestion made here that their relative quantity is an effect of aggregation between the two types of molecules themselves or among the pulse-labeled RNA and the two sizes of ribosomal RNA.

The separation between s-RNA and the other RNA has proved useful, however, for kinetic studies. Studies with [^{14}C]uracil and ^{32}P showed a delay in incorporation of label into s-RNA relative to the other RNA of about 1–2 min. Rather than being the result of a precursor pool of nucleotides, this delay seems to be a reflection of the turnover of D-RNA. The apparent difference between the delays experienced in [^{14}C]uracil and ^{32}P labeling may be attributable to the special features of uracil incorporation³⁹. Thus some of the [^{14}C]uracil could enter s-RNA directly so that the

initial rate of entry is not zero and the delay resulting from the utilization of nucleotides derived from D-RNA breakdown is apparently reduced.

A unified picture of the flow of label into RNA would be the following. The initial flow is accounted for by a one-third entry into D-RNA and the remaining two thirds into R-RNA. The former is degraded and the nucleotide material used to some extent for s-RNA and DNA synthesis. Of the nucleotides originally entering D-RNA some would serve to make s-RNA (20 % of the total RNA) and DNA, about 15 % of the cell nucleic acid, and possibly R-RNA. If D-RNA were degraded to nucleoside 5'-diphosphates or monophosphates⁴¹ the conversion of D-RNA to DNA could proceed by the mechanism described by COHEN *et al.*⁴². Thus rather than being an obligatory precursor of R-RNA¹⁶ the D-RNA is the precursor of s-RNA and DNA and only to a more limited extent that of R-RNA. This picture would fit all the kinetic experiments and finds support from the studies of chloramphenicol inhibition.

The presence of chloramphenicol in the growth medium during the incorporation of [¹⁴C]uracil into RNA removes the kinetic delay of the entry of label into s-RNA. Thus more ¹⁴C enters s-RNA and DNA directly. It is unlikely that the removal of the delay by chloramphenicol can be adequately explained by changes in a small molecule precursor pool feeding s-RNA. This effect has been shown to be associated with an accumulation of D-RNA. Although the rate of synthesis of D-RNA remains the same, its degradation is markedly reduced. The conversion of "chloramphenicol RNA" to soluble material when the antibiotic is removed^{37,43} may reflect the renewal of the degradation process, although it is not clear whether both the D-RNA and R-RNA fractions are lost.

With a knowledge of the number of ribosomes per cell (10^4), it is possible to estimate the maximum number of ribosomes which can have D-RNA associated with them. Taking the quantity of D-RNA as about 1/3 of the total 14-S fraction, itself about 2.7 % of the total RNA⁹, and its average mol. wt. as $2 \cdot 10^5$, one obtains the result that less than one ribosome in ten has a D-RNA molecule⁴⁴.

There are now many lines of evidence suggesting that the template RNA has a D-RNA nucleotide composition. It is the only RNA which is synthesized in phage-infected cells¹ and D-RNA synthesized *in vitro* stimulates cell-free protein synthesis⁸. It is therefore reasonable to suppose that the present estimate of D-RNA in the cell is also an upper limit of the amount of template RNA.

Knowledge of the rate of protein synthesis under these conditions enables the calculation of the maximum period of association between ribosomes, template and nascent protein molecule. The rate of protein synthesis in the conditions used for cell growth is about 1 μ mole of amino acids/g cells/sec incorporated into protein, and the quantity of D-RNA is about 6 μ moles/g cells. Assuming that all the D-RNA is template material and that the coding ratio of nucleotides for amino acid specification is 3 (see ref. 45), the template can be associated with each growing protein chain for only some 2 sec. The apparent rate of synthesis of D-RNA, based on tracer incorporation is 0.04 μ mole/g cells/sec. If this is in fact the true rate of synthesis it is not high enough to allow its destruction after each round of protein synthesis.

Kinetic studies indicate that the lifetime of the D-RNA molecules is very similar to that of the total 14-S fraction. Thus the average lifetime of template RNA molecules is probably of the order of 2.5 min. This is in agreement with measurements of the lifetime of the enzyme-forming unit apparent from studies of the induction of β -

galactosidase^{46,47} and other enzymes⁴⁶. Template molecules would be involved in the synthesis of as many as 75 protein molecules.

In spite of the inefficiency of cell-free protein synthesis, it is already clear that polyuridylic acid can synthesize more than one equivalent of polyphenylalanine before it is degraded⁴⁸. In reticulocytes the template RNA is apparently stable⁴⁹.

Although these calculations indicate that template-RNA molecules can be in contact with the site of synthesis of any one protein chain for only some 2 sec, it is evident from studies of protein synthesis under similar conditions of growth^{36,50} that ribosomes hold 5–10 sec supply of nascent protein. This may imply that the template RNA leaves the ribosome before the completed protein. This would allow it to initiate the synthesis of proteins on other ribosomes. The completion of the protein molecule and the acquisition of its secondary structure may thus be carried out in the absence of template. The association of one template molecule with more than one ribosome makes it possible to reconcile the limited amount of template with the fact that protein synthesis appears to be proportional to the total number of ribosomes^{51–54}.

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Comment. The existence of two components of different composition in the rapidly labeled RNA is confirmed by their separation described in III.B.6. The same conclusion has been reached in studies of the RNA synthesis in *Rhodospseudomonas spheroides* (A. M. Haywood, E. D. Gray, and E. Chargaff, *Biochim. Biophys. Acta*, 61, 155, 1962) and yeast (Y. Kitasume and M. Yeas, in press, 1963). In each of these reports it was suggested that the RNA consists of approximately equal parts of messenger-like RNA with a base composition close to that of the DNA and ribosomal RNA. On the other hand, Ishihama et al. (*J. Mol. Biol.*, 5, 251, 1962) claim that all rapidly labeled RNA in *E. coli* has a DNA-like composition. In HeLa and mouse L tissue culture cells also most of the rapidly labeled RNA is ribosomal precursor and only part has the characteristics of messenger (K. Scherrer, H. Latham, and J. E. Darrell, *Proc. Natl. Acad. Sci. U. S.*, 49, 240, 1963; A. V. Rake and A. F. Graham, *Biophys. J.*, in press, 1964). Brian J. McCarthy.

III.B.6 A General Method for the Isolation of RNA Complementary to DNA

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During the past few years evidence has accumulated that a class of RNA molecules which resemble DNA in base composition is synthesized in living cells. It has been proposed that this RNA is an intermediary in the transfer of genetic information from DNA to protein.¹ It is therefore of importance to have available a general method for the purification of such RNA molecules in order to study their chemical character and information content. Hall and Spiegelman² showed that T2-specific RNA could be hybridized with heat-denatured T2 DNA and the hybrid isolated by cesium chloride density gradient centrifugation. With this method Hayashi and Spiegelman³ were able to demonstrate small amounts of DNA-like RNA in nongrowing bacteria. Base analyses of rapidly labeled RNA strongly suggest the existence of complementary RNA in several species of growing bacteria^{4, 5} but attempts to purify such molecules have been only partially successful.⁵

Bautz and Hall⁶ have made a notable advance toward purifying RNA molecules with a base sequence complementary to DNA. They used phospho-cellulose ace-

tate to immobilize denatured T4 phage DNA. The mechanism proposed for this attachment involved covalent bond formation between the glucosylic hydroxyls of the DNA and phosphate groups on the cellulose. The reaction therefore appeared to be limited to glucosylated DNA known to occur only in some bacteriophages. Incubation of RNA solutions with this preparation immobilized some RNA by specific hybridization. Noncomplementary RNA could then be washed away and complementary RNA reclaimed after decomposition of the hydrogen bonds between RNA and DNA.

Investigation of this reaction has led to a method for immobilizing any high molecular weight DNA by physical entrapment in cellulose acetate gels, or more readily, in agar gels. These preparations of immobilized DNA can form hydrogen bonds with complementary molecules. This paper describes the preparation of DNA-cellulose acetate and DNA-agar gels and their application to the isolation of complementary RNA.

Methods.—(a) *Preparation of DNA-cellulose acetate:* DNA was dialyzed successively against water, formamide, and anhydrous pyridine.⁷ 2 gm cellulose acetate (Fisher Scientific Co., cellulose diacetate, "acetone soluble") were dissolved in 10 ml boiling pyridine and precipitated by the dropwise addition of 15 ml water. The precipitate was gathered on a glass rod and dissolved in 15 ml boiling pyridine containing 10 mg DNA. 7.5 gm dry cellulose powder were then added to soak up the liquid, and the crumbly mixture allowed to cool. One molar NaCl solution was poured onto the mass and gelation allowed to occur. When the gel was hardened it was ground in a mortar and washed with $2 \times \text{SSC}$ ($\text{SSC} = 0.15 \text{ M}$ sodium chloride, 0.015 M sodium citrate) at 60°C . Trapping of DNA was greater than 70 per cent.

(b) *Preparation of DNA-agar:* 300 mg of agar (Difco Special Agar-Noble) were dissolved in 5 ml of water at 100°C . 5 mg DNA in 5 ml $0.01 \times \text{SSC}$ were heated to 100°C for 5 min and poured into the agar. The hot solutions were thoroughly mixed and poured into an empty 250 ml beaker in an ice water bath. When the gel had hardened it was forced through a 35 mesh screen. The gel particles were washed with $2 \times \text{SSC}$ at 60°C . Trapping of DNA was essentially quantitative.

(c) *DNA:* Bacterial DNA was prepared by the method of Marmur⁸ and bacteriophage DNA according to Grossman *et al.*⁹ Thymus DNA was obtained from Worthington.

(d) *Preparation of phage specific RNA:* Strain BB of *E. coli* growing in a tris/glucose medium¹⁰ at 37°C was infected with T2 phage at a multiplicity of 10. Phage RNA was labeled through the addition of P^{32} ($20 \mu\text{C}/\text{ml}$) 5 min after infection. The cells were chilled 5 min later by pouring onto crushed frozen medium. The cells were washed, frozen, and lysed with lysozyme.² DNAase ($20 \mu\text{g}/\text{ml}$) was added for 2 min, followed by 1 per cent sodium dodecyl sulfate. RNA was extracted with phenol and purified by means of a Na^+ Dowex-50 Sephadex G25 column.⁶

(e) *Preparation of bacterial RNA:* RNA either pulse labeled with P^{32} , or randomly labeled with P^{32} and pulse labeled with C^{14} -uracil, was prepared from growing *Proteus vulgaris* as previously described.^{5, 11} It was purified as described above for phage specific RNA.

(f) *Hybridization of RNA with immobilized DNA:* Approximately $50 \mu\text{g}$ of RNA in 1 ml or less of $2 \times \text{SSC}$ were incubated at 60°C with DNA-cellulose acetate or DNA-agar containing 0.5 mg DNA, either in a thick slurry in a screw top vial, or in a chromatograph tube heated by circulating water. The preparations heated in vials were then transferred to chromatograph tubes for subsequent operations. The column was washed with six to ten 5-ml aliquots of $2 \times \text{SSC}$ and the aliquots separately collected. Recovery of the remainder of the labeled RNA was effected by washing with six to ten 5-ml aliquots of $0.01 \times \text{SSC}$ which were also individually collected. The flow rate was approximately 1–2 ml per minute. Carrier RNA was added to the fractions and the total RNA precipitated with 5 per cent trichloroacetic acid. The precipitates were collected on membrane filters and assayed for radioactivity.¹¹

(g) *Base compositions* were determined as described by Midgley.¹⁰

Results.—*Hybridization with DNA-cellulose acetate:* Phage T2 DNA in cellulose acetate (0.5 mg DNA in 1 gm cellulose) was incubated overnight at 60°C with 50

μg of the RNA labeled with P^{32} after phage infection. The preparation was then washed and the hybrid decomposed as described above. It was found that about 10 per cent of the phage specific RNA had been hybridized. When this RNA preparation was incubated with *P. vulgaris* — DNA in cellulose acetate or with a cellulose acetate preparation lacking DNA, 1–3 per cent of the phage specific RNA was eluted at the low salt concentration. When 20 μg of *P. vulgaris* P^{32} pulse labeled RNA were incubated with a homologous DNA-cellulose preparation, 17 per cent of the P^{32} label was affixed and this could be removed by lowering the salt concentration. The incubation of *P. vulgaris* RNA with T2-DNA, or blank, —cellulose acetate fixed a smaller fraction of the P^{32} . When 200 μg *P. vulgaris* RNA randomly labeled with P^{32} and labeled for 2 minutes with H^3 -uracil were incubated with the homologous DNA preparation, about 20 per cent of the H^3 , and 2 per cent of the P^{32} were fixed. Thus, this procedure yielded some purification of the pulse labeled RNA, and the hybridization process appeared to be specific for both T2-specific and *P. vulgaris* RNA. However, the yields of the apparent hybrids were disappointingly low, and the hybridized material was accompanied by a relatively high background of nonspecifically adsorbed nucleic acid. About 2 μg of RNA were adsorbed per gram of cellulose even when DNA was not present. The low yields of hybrid might well have been a result of trapping DNA so tightly that it was unavailable for hybridization. In support of this notion DNAase treatment of the DNA-cellulose acetate removed only about 10 per cent of the DNA in one-half hour at 20°C and only about 50 per cent overnight. In contrast DNAase treatment for one-half hour at 20°C removed over 80 per cent of the DNA in DNA-agar prepara-

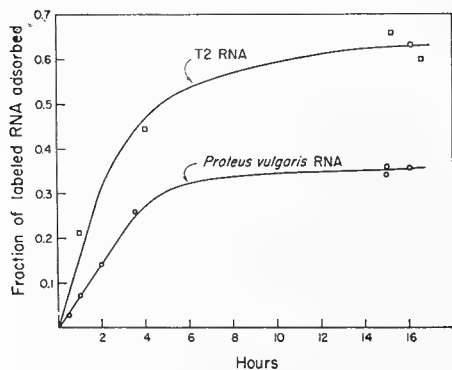


FIG. 1.—The rate of adsorption of RNA to a column of DNA-agar gel. 50 μg of P^{32} labeled RNA prepared from T2-infected *E. coli* or RNA of *P. vulgaris* labeled with P^{32} for one min were incubated in $2 \times \text{SSC}$ for various times at 60°C in a 2 cm column of agar containing 0.5 mg of the corresponding DNA. At times indicated the unadsorbed RNA was removed by washing with six 5-ml fractions of $2 \times \text{SSC}$. The adsorbed RNA was recovered by elution with six 5-ml portions of $0.01 \times \text{SSC}$ and the fraction of the labeled RNA adsorbed was recorded.

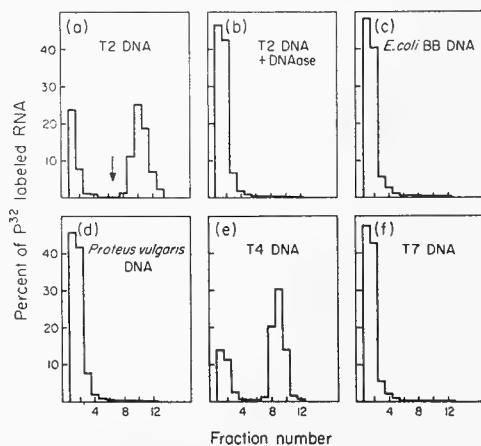


FIG. 2.—The specificity of the adsorption of T2-specific RNA to columns of DNA-agar gel. 50 μg of RNA prepared from T2 infected cells labeled after infection with P^{32} were incubated in $2 \times \text{SSC}$ for 15 hr with 1 gm of agar gel containing 0.5 mg of DNA from various sources. Elution as in Fig. 1. Eluting solution changed to $0.01 \times \text{SSC}$ at fraction 7. Fractions were assayed for the percentage of the P^{32} labeled RNA. (a) Agar containing T2 DNA. (b) T2 DNA trapped in agar and treated with DNAase at 20 $\mu\text{g}/\text{ml}$ for 2 hr at 25°C . (c) *E. coli* BB DNA. (d) *P. vulgaris* DNA. (e) T4 DNA. (f) T7 DNA.

tions. Hence, the DNA in the agar appeared to be more readily available for contact with macromolecules.

Formation of RNA-DNA hybrids in agar: Figure 1 shows the time course of hybrid formation at 60°C in the homologous reactions: T2-specific RNA and T2 DNA-agar, and pulse labeled *P. vulgaris* RNA and *P. vulgaris* DNA-agar. In each case the reaction progresses smoothly and slowly as would be expected for a hybridization process. Under the conditions used about one-third of the *P. vulgaris* pulse labeled RNA and about two-thirds of the phage specific RNA can be hybridized. The latter result may be compared with that of Bautz and Hall⁶ who found that 80 per cent of the T4-specific RNA could be hybridized with homologous DNA-cellulose. For convenience overnight incubations have been generally employed. The greater part of the reaction is, however, complete much earlier, especially if incubation is carried out in a column so that the concentration of reactants is as high as possible. Incubation as a thick paste in a vial produces rates of reaction lower by not more than a factor of two.

Specificity of hybrid formation: Figure 2 shows that the fixation of T2-specific RNA to DNA-agar depends upon the DNA. Thus *E. coli* BB DNA-agar (the host strain used for culture of T2 phage), *P. vulgaris* DNA-agar, and T7 phage DNA-agar do not allow fixation of the T2-specific RNA. No reaction occurs with preparations treated with DNAase. On the other hand, fixation is relatively efficient with the homologous T2 DNA preparation as well as with DNA-agar of the closely related bacteriophage, T4. A rerun of the adsorbed RNA from Figure 2(a) gave 70 per cent of the radioactivity in the back peak.

Figure 3 shows the specificity of reaction for the case of pulse labeled *P. vulgaris* RNA. This RNA hybridizes with the homologous DNA-agar but not with the unrelated DNA-agar of phage T2 which has the same base composition as *P. vulgaris* DNA. There is no reaction with the DNA-agar of *Pseudomonas aeruginosa* or calf thymus. Again a rerun of the back peak of Figure 3(a) allowed 70 per cent of the radioactive RNA to appear in the back peak.

Base composition of the adsorbed RNA: Analyses were made of the base compositions of the adsorbed and unadsorbed fractions of both the T2-specific RNA preparation and the one-minute P³² pulse labeled *P. vulgaris* RNA (Table 1).

In the case of the T2-specific RNA it is clear that essentially all of the labeled RNA has a composition close to that of the virus DNA. The composition of the unadsorbed material is not significantly different from that in the back peak. The disparity between the contents of cy-

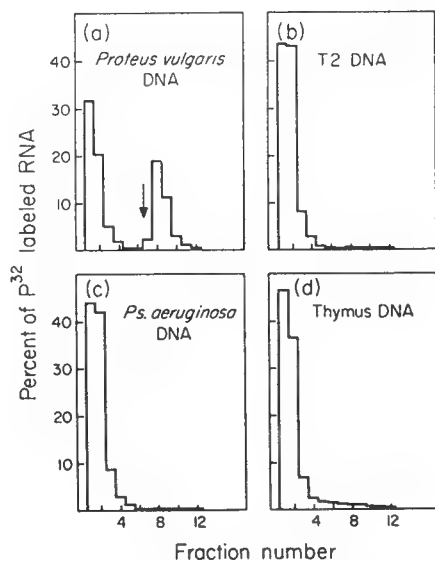


FIG. 3.—The specificity of the adsorption of RNA from *P. vulgaris* labeled with P³² for 1 min to columns of DNA-agar gel. Conditions as for Fig. 2. (a) Agar containing *P. vulgaris* DNA. (b) T2 DNA. (c) *Ps. aeruginosa* DNA. (d) Calf thymus DNA.

tidylic acid and guanylic acid has also been noted by other workers.^{6, 12} Evidently the conditions of the incubation are not sufficient to hybridize more than about 70 per cent of the specific RNA.

The base composition of the RNA synthesized in the first 3 minutes after the addition of P^{32} to growing bacteria is intermediate between that of the bacterial DNA and ribosomal RNA.^{4, 5} This could result from the entry of P^{32} into 2 different types of RNA molecule, one DNA-like (D-RNA) and the other ribosomal. Even with the shortest (10 seconds) exposure to P^{32} there seemed to be a partition of the label between 2 types of newly synthesized molecule.⁵ Fractionation of the pulse labeled RNA of *P. vulgaris* on the *P. vulgaris* DNA-agar column demonstrates the validity of this interpretation. The labeled RNA is separated into 2 components of very different composition. The adsorbed material has a composition closely resembling that of the DNA and the unadsorbed RNA is very like ribosomal RNA. The rapidly labeled RNA would therefore appear to be only about 30 or 40 per cent D-RNA and the remainder precursor of ribosomal RNA.¹³

TABLE 1
BASE COMPOSITION OF P^{32} LABELED RNA SEPARATED BY DNA-AGAR

	C	Mole Fraction		U(T)	% GC
		A	G		
T2-specific RNA:					
Unadsorbed RNA 34%	18.4	28.6	24.1	28.9	43
Adsorbed RNA 66%	17.4	31.0	21.9	28.7	39
Bacteriophage DNA	18	32	18	32	36
Pulse labeled <i>P. vulgaris</i> RNA:					
Unadsorbed RNA 65%	22.5	23.3	32.1	22.1	55
Adsorbed RNA 35%	19.8	30.1	20.9	29.2	41
Purified 14S RNA fraction of <i>P. vulgaris</i> labeled for 5 min with P^{32} (ref. 5)	22.7	26.7	27.6	23.0	49
<i>P. vulgaris</i> ribosomal RNA ¹⁰	21.7	26.2	31.4	20.7	53
<i>P. vulgaris</i> DNA	19	31	19	31	38

Kinetics of labeling of DNA-like RNA: The separation of pulse labeled bacterial RNA into 2 components and the consequent purification of the D-RNA makes it possible to study the rate of synthesis of the latter component. Previously all studies of the kinetics of labeling of the rapidly labeled 14S fraction have been concerned with a mixture of ribosomal RNA precursor (R-RNA) and D-RNA.^{11, 14} As a result estimates of the quantity and half-life of the D-RNA molecules could be made only by correlation of the results of kinetic studies and the changes in base composition of the total RNA with time of labeling.⁵ The present method permits the measurements to be made directly.

Cells of *P. vulgaris* were grown in C medium containing P^{32} for 3 generations. The generation time was 72 minutes. C^{14} -uracil was then added and samples taken for the isolation of RNA after 1, 2, 3, 5, 7, and 10 minutes. 30 μ g of each of the RNA samples were then incubated with agar gel containing 0.5 mg of *P. vulgaris* DNA for 15 hours at 60°C. The fractionation of the 1-minute sample is shown in Figure 4. It is evident that about one-third of the C^{14} -labeled RNA is in the hybrid peak and is accompanied by only a small fraction of the total RNA as represented by the P^{32} label. The C^{14} and P^{32} radioactivity in the front and back peaks were computed for each time point. After normalization to the same size sample of RNA by means of the total P^{32} eluted, the C^{14} radioactivity in the total

RNA and in the adsorbed peak were plotted (Fig. 5). A small correction was applied to the latter series of numbers to account for the small amount of ribosomal RNA appearing in the back peak. This may be due to specific hybridization between ribosomal RNA and DNA.^{15, 16} The correction can be easily estimated from a knowledge of the total RNA adsorbed in the hybrid peak as measured by the P^{32} radioactivity.

The rate of entry of C^{14} -uracil into total RNA produces a straight line through the origin as in *E. coli*.¹¹ Apparently the initial rate of entry of label into the D-RNA accounts for about one-third of this flow. The entry of C^{14} -uracil into D-RNA soon levels off indicating an average lifetime of these molecules of about 2 minutes. Estimation of the quantity of D-RNA from these kinetics is made somewhat inaccurate by the large correction at late times, but the curve suggests an amount corresponding to the RNA synthesized during 1 minute; i.e., approximately 1 per cent of the total RNA content.

Discussion.—Once it is realized that single-stranded DNA can be immobilized by trapping in a gel, a large number of variants on the methods described above become available. The long length of the DNA molecules makes it possible to confine them in a three-dimensional structure even where the holes in the structure are relatively large. Of the two types of gels here employed it seems clear that the agar gels are the most permeable. This is certainly true with respect to availability to the action of DNAase. DNA trapped in cellulose acetate by the method outlined above showed disappointingly low efficiency although this is not true of the preparations used by Bautz and Hall.⁶

In addition there remains the possibility that the DNA in the preparation of Bautz and Hall is bound covalently to the cellulose as they indicated. In our experience, however, very similar quantities of DNA are bound to the column whether or not the conditions for phosphate ester formation are met. Thus, the yield is apparently unaffected by the presence of the condensing agent, dicyclohexylcarbodiimide, and certainly fixation is not limited to those bacte-

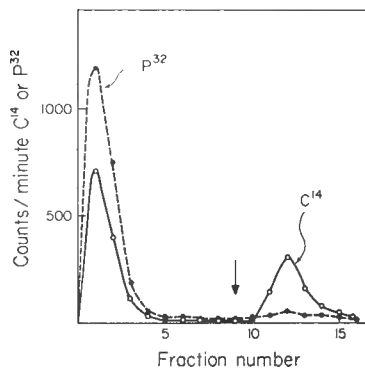


FIG. 4.—The adsorption of *P. vulgaris* RNA labeled for 3 generations with P^{32} and for 1 min with C^{14} -uracil to a column of DNA-agar gel. Incubation 15 hr at 60°C . Elution with eight 5-ml portions of $2 \times \text{SSC}$ followed by eight 5-ml portions of $0.01 \times \text{SSC}$.

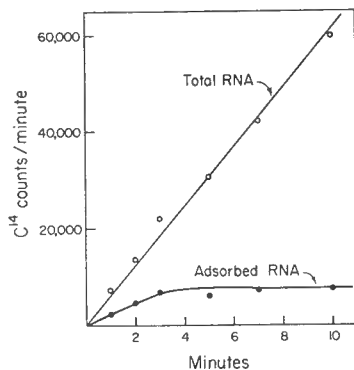


FIG. 5.—The rate of synthesis of RNA adsorbable by a DNA-agar gel column. 6 samples of RNA from *P. vulgaris* cells grown for 3 generations in P^{32} with a generation time of 72 min were taken after exposures of 1, 2, 3, 5, 7, and 10 min to C^{14} -uracil. Each was incubated with an agar gel containing *P. vulgaris* DNA $30 \mu\text{g}$ RNA/0.5 mg DNA (see Fig. 4). The C^{14} cpm in the total RNA and the adsorbable material was normalized for the amount of RNA employed by means of the P^{32} cpm and plotted against time. Data from Fig. 4 and five additional analyses.

riophage DNA's having glucosylated residues containing reactive hydroxyl groups.

In general, however, it appears that preparations of DNA agar have advantages over those in cellulose. The method of preparation has the great advantages of ease and rapidity and the material prepared has properties suitable for packing in a column and rapid equilibration with the ionic environment. The gel network is a very open structure since Steere and Ackers¹⁷ have shown that even gels made from 4 per cent agar have holes large enough to accommodate southern bean mosaic virus (molecular weight, 6.6×10^6). There is, however, a failing of the agar materials which may be of consequence; the 3 per cent agar preparations are not resistant to temperatures above 70°C and in cases where higher temperatures are necessary for the formation or dissociation of hybrid molecules, other means of immobilizing DNA must be employed. Probably other gels such as polyacrylamides or related compounds will prove useful for this purpose.

The high specificity shown in the reaction between RNA and DNA immobilized in agar suggests that relatively long regions of complementary sequences are necessary for attachment under the conditions of these experiments. Thus the reaction does not occur between molecules of DNA and RNA derived from different sources, e.g., T2 and *P. vulgaris* nucleic acids, even though they have the same average base composition. Likewise, bacterial nucleic acid has no reaction with thymus DNA which is presumably highly diversified in base sequences. It is probable that the high temperature maintained throughout the separation precludes adventitious pairing of short regions to form spurious hybrids. It is not clear at the moment, however, how long the paired regions must be to survive incubation and washing at 60°C.

It is of considerable interest that there exists a high degree of cross reaction between the RNA of T2-infected cells and the DNA of T4 phage, while no reaction with phage T7 DNA is noted. Clearly, the method here employed appears to have potential for exploring quantitatively the genetic relatedness among species. In this connection we have preliminary results indicating the feasibility of such studies with several bacterial species.¹⁶ Schildkraut *et al.*¹⁸ have already demonstrated formation of interspecific hybrid DNA-DNA molecules by means of the cesium chloride method. The ease and economy of the present method may make possible a quantitative taxonomy based on homologous sequences of nucleotides in nucleic acids.

Application of the method to the rapidly labeled RNA of bacteria results in considerable clarification of some questions relating to messenger-RNA. The labeled RNA, which is all present in the 14S peak in extracts made after short exposures to P³², can be cleanly separated into 2 types of molecule easily distinguishable by their base composition. The 14S RNA comprises 3 per cent of the total RNA.¹¹ This establishes that at least half of that RNA commonly referred to as "messenger" as judged by its sedimentation coefficient and its high rate of labeling¹⁴ is actually ribosomal RNA presumably in some precursor stage before entering ribonucleoprotein particles.¹¹ Moreover, the fact that such a high proportion of the P³² enters ribosomal RNA in 1 minute shows that the rate of synthesis of the D-RNA fraction cannot be higher than that of the bulk RNA. Rather, it seems that approximately one-third of the total cellular synthesis of RNA is in the form of D-RNA. The final level attained by the C¹⁴-uracil in the D-RNA peak

demonstrates that about 1 per cent of the cellular RNA of growing cells is in this form. This estimate depends upon quantitative removal of the D-RNA by the DNA agar column. The base analysis of the separated R-RNA and D-RNA (Table 1) shows that the removal was highly effective.

Measurement of the rate of labeling of the hybridizable RNA with C¹⁴-uracil indicates an average lifetime of these molecules of about 2 minutes which is similar to that of the β -galactosidase forming unit.^{19, 20} As pointed out in more detail elsewhere,⁵ such a period of activity on the part of template or messenger-RNA molecules, which should form part, at least, of the D-RNA fraction, would allow the direction of the synthesis of perhaps 50 protein molecules. The participation of template RNA in protein synthesis would therefore appear to be catalytic rather than stoichiometric.

Summary.—Methods are described for the immobilization of single-stranded DNA by trapping in gels of cellulose acetate or agar. The immobilized DNA retains the ability to form specific hybrids with complementary RNA. This provides a general method for the isolation of RNA molecules having sequence homology with DNA molecules from any given source. The specificity and kinetics of the hybridization reaction are examined, and the method is illustrated by a study of the rate of synthesis of complementary RNA in *Proteus vulgaris*.

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Comment. This paper will appear again in a companion volume which will assemble the Biophysics Section's published work on nucleic acid interactions.

Ellis T. Bolton.

C. Related Papers

III.C.1 Stability of Ribonucleoprotein Particles of Escherichia coli

(Reprinted, by permission, from Microsomal Particles and Protein Synthesis, pp. 18-21, Pergamon Press, New York, 1958.)

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Investigations concerned with the structure and function of ribonucleoproteins of microorganisms require particle preparations that are representative, reproducible, and stable. This report presents some results of exploratory studies in which the analytical ultracentrifuge was used to assess the influence of various suspending media on the ribonucleoproteins of *Escherichia coli*.

METHODS

E. coli, B (ATCC 11303) harvested during the exponential phase of growth in a glucose-salts culture medium¹ was used for all studies. The bacteria were washed and resuspended (25 mg dry weight of bacteria per milliliter) in appropriate buffer solutions and disrupted by means of a modified French pres-

¹ The composition of C medium and other culture conditions may be found in Roberts et al., *Studies of Biosynthesis in Escherichia coli*, Carnegie Inst. Wash. Publ. 607, Washington, D. C., 1955.

sure cell² operated at approximately 10,000 psi. Breakage of the bacteria by this means is essentially complete. The resulting bacterial juices were examined in the analytical ultracentrifuge (Spinco, Model E) as soon as practicable (about 30 minutes after rupture), or after various periods of storage at 4° C. The centrifuge was routinely brought up to speed in 6 to 7 minutes and held at about 60,000 rpm for the duration of the run.

RESULTS

Figures 1 to 4 are illustrative sedimentation diagrams showing that the pattern of rapidly sedimenting components varies in accord with the kind of suspending medium used. Figure 1 compares the sedimentation behavior of the components in extracts prepared from bacteria broken in 0.01 *M* Tris–0.004 *M* succinic acid–0.005 *M* magnesium acetate buffer (*pH* 7.6, “TSM”), in 0.01 *M* Tris–0.004 *M* succinic acid (“TS”), or in TSM+0.07 *M* phosphate (*pH* 7.6). Values spotted along the abscissa are approximate apparent sedimentation coefficients. It is evident from this comparison that more, and larger, components are observed when magnesium has been included in the buffer and also that phosphate abolishes the more rapidly sedimenting materials. Whether the effect of phosphate is specific or whether the result is due to an increased ionic strength of the medium is not known. The sharp spike characteristic of highly polymerized deoxynucleic acid (DNA)³ is missing from these diagrams, although it is readily observed in juices prepared by breaking *E. coli* as a result of lysozyme treatment and osmotic shock. In spite of this finding, three-quarters of the ultraviolet-absorbing substance and one-seventh of the protein of *E. coli* disrupted in the TSM medium may be sedimented in the preparative rotor (100,000*g*, 90 minutes).

Figure 2 shows that juices prepared by pressure cell disruption maintain constant sedimentation diagrams for at least 20 hours. If, however, sodium ethylenediaminetetraacetate (EDTA, 0.1 *M*, *pH* 7.6) is added to the bacterial extracts, all components greater than about 20 *S* disappear. This occurs whether

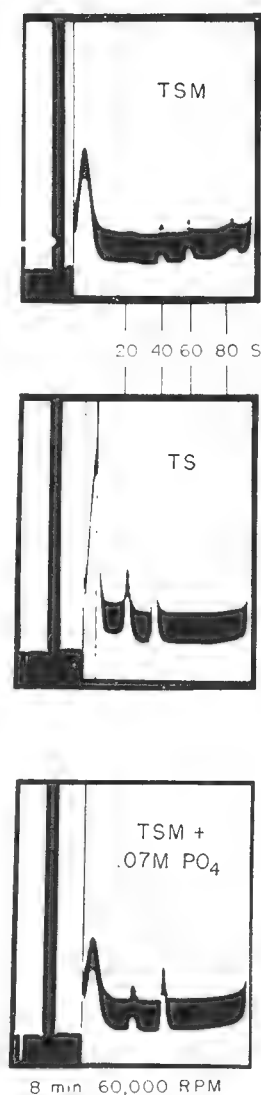


Fig. 1. Sedimentation diagrams of *E. coli* disrupted in various buffer solutions. The concentration of the bacterial juices differed among the runs.

² C. S. French and H. W. Milner, *Methods in Enzymology I*, Academic Press, p. 65. A similar device is marketed by the American Instrument Company, Silver Spring, Maryland.

³ See, for example, the sedimentation diagrams reported by W. Gilchrist and R. Bock, S. Dagley and J. Sykes, and J. Wagman reported in the present volume.

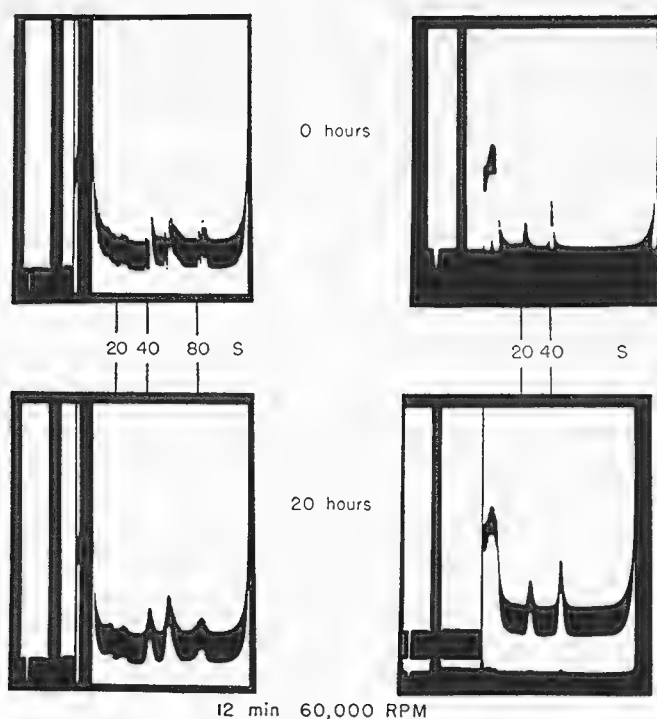


Fig. 2. Influence of storage at 4° C. The suspending buffers (TSM, left; TS, right) also contained 0.25 M sucrose, although subsequent runs have shown that sucrose has no effect on the pattern of components.

or not magnesium is included in the original suspending medium, as figure 3 demonstrates. In addition, a markedly decreased (<10 per cent) ultraviolet absorption occurs in the 100,000g-1 hour pellets (preparative rotor) when EDTA has been added to the bacterial juices. Figure 4 shows that DNAase (2 µg/ml) has little, if any, effect upon the number and size of the rapidly sedimenting materials, whereas RNAase (approximately 10 µg/ml) removes these components.

SUMMARY AND CONCLUSIONS

Pressure cell disruption of *E. coli* at pH 7.6 in magnesium-containing solutions of low ionic strength (e.g., 0.01 M Tris-succinate) releases high-molecular-weight components which range from 20 to 80 S. These components "fall apart," i.e., become elements having sedimentation coefficients less than about 20 S, when a chelater, EDTA, or the enzyme ribonuclease is allowed to act upon them. DNAase, sucrose, or cysteine exerts no apparent effect on either the number of components or their relative quantities. Nearly all (>80 per cent) of the ribonucleic acid and about one-seventh of the protein of *E. coli* can be sedimented in a preparative rotor under optimum conditions (TSM, 100,000g, 90 minutes). No RNA and only a trivial amount of protein can be sedimented after EDTA or ribonuclease treatment. Hence, it may be con-

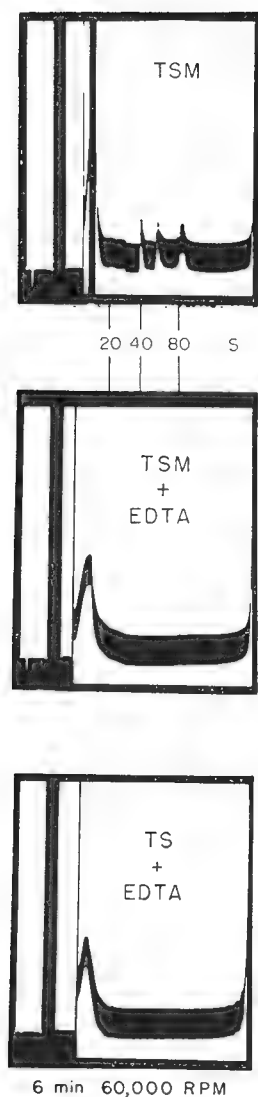


Fig. 3. Effect of EDTA on the sedimentation diagrams of *E. coli* juice. The two lower diagrams are from preparations containing one-half as much material as those for the upper pattern.

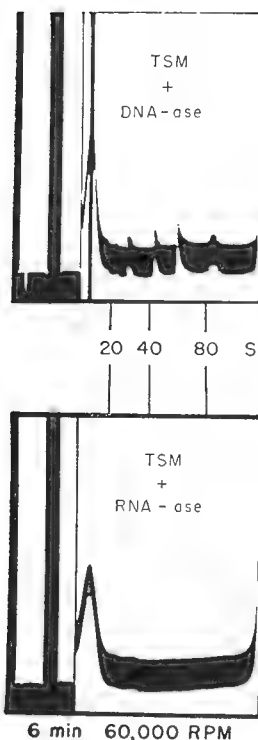


Fig. 4. Effect of nucleases on sedimentation diagrams. The lower pattern is from a preparation one-half as concentrated as that of the upper diagram.

cluded that the bulk of the high-molecular-weight components of *E. coli* is composed of ribonucleoproteins held together in a fashion in which divalent cation(s) (probably Mg^{++}) and the integrity of ribonucleic acid play important roles. Thus, in certain physical and chemical attributes the "ribosomes" (ribonucleoprotein particles) of *E. coli* resemble constitutive elements of the cytoplasm of other bacteria, and also of yeast, plants, and mammals.

Comment. The work described above was actually carried out at the Rocky Mountain Laboratory, Hamilton, Montana, during the delightful summer of 1957. Soon thereafter we found that F. C. Chao and H. K. Schachman had already reported (Arch. Biochem. Biophys., 61, 220, 1956; F. Chao, ibid., 70, 426, 1957) on the effect of Mg^{++} on the stability of yeast ribosomes, and Petermann et al. (Microsomal Particles and Protein Synthesis, pp. 70-75) showed the effect for rat liver ribosomes. Thus, as is now well known, sensitivity to divalent cations appeared to be a general property of ribosomes. Ellis T. Bolton.

III.C.2 Fractionation of Escherichia coli for Kinetic Studies

(Reprinted, by permission, from Microsomal Particles and Protein Synthesis, pp. 84-94, Pergamon Press, New York, 1958.)

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A single cell of the bacterium *Escherichia coli* contains roughly 10,000 ribosomes (microsomal particles). If the cells are broken open and their contents are examined, the analytical centrifuge shows a series of peaks with sedimentation constants of roughly 20, 30, 40, 60, and 80 S [1-8]. The existence of these particles in such variety and in such large numbers immediately provokes a number of questions. Is the rapid growth rate of *E. coli* a consequence of the high proportion (25 per cent) of the cellular material that is organized into ribosomes? In other words, are ribosomes the sites of protein synthesis in *E. coli*? If so, what size of particle is active in protein synthesis? How are the particles themselves synthesized? Do the different sizes of particles represent different stages in the growth of a particle?

Eventually answers will be found for these questions, but not easily. It will be necessary to know the composition of the particles—the composition of the individual classes of particles, not just the composition of a pellet containing an assortment of particles plus other material. Also the kinetics of isotope incorporation will have to be studied. It will not be sufficient simply to deal with the microsomal fraction, the 100,000g pellet; rather, the individual groups of particles will have to be sorted out and measured. Suppose, for example, that one size of particle is the precursor of another. Kinetic measurements will show this clearly if the two groups can be resolved; kinetic measurements of both groups lumped together in a pellet will show nothing.

Chemical fractionation of *E. coli* gives good separation between the different classes of compounds, and it is easy to show by kinetic measurements of the incorporation of radioactive compounds that the small molecules serve as

precursors of the large ones. In contrast, the simple separation into cell wall, microsome, and soluble fractions is not sufficient to reveal clearly any precursors or products among the macromolecules. A further fractionation of the microsome pellet is required.

Pellets of somewhat greater homogeneity can be obtained by choosing an appropriate centrifuging schedule. The material that sediments in 15 minutes at 100,000g is richer in the large particles than the pellet obtained by centrifuging down (2 hours at 100,000g) material which stayed in suspension during three successive 15-minute periods at 100,000g. The composition of the pellet also varies; the early pellet contains nearly twice the lipid and protein per unit of nucleic acid. This approach, however, shows no promise of giving adequate fractionation.

A somewhat better fractionation can be obtained by using the swinging bucket head for the Spinco Model L centrifuge. Microsome pellets are resuspended and layered on top of a sucrose gradient. After a period of centrifugation, layers are taken off with a pipet. This technique is adequate to show marked differences in the distributions, depending on the initial material. Figure 1 shows one curve for a resuspended pellet composed mostly of large (80 S) particles; another for the smaller particles (20 to 40 S) that result if magnesium is lacking [8]; and a third, for the nonsedimenting material. The analytical

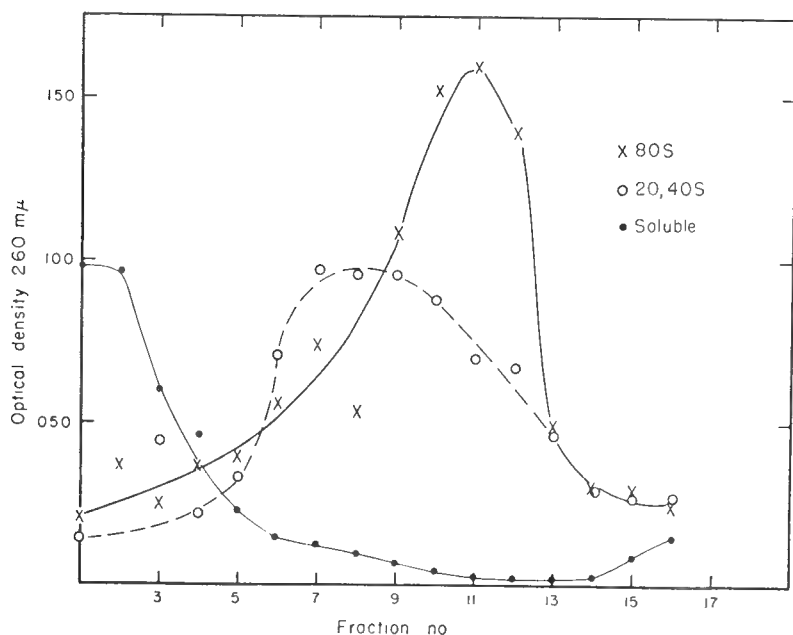


Fig. 1. Fractionation of particle preparations using the swinging bucket centrifuge. Five-tenths milliliter of suspension is placed on top of 4.5 ml sucrose gradient in the centrifuge tube. After 45 minutes at 100,000g, 0.3-ml fractions are taken off from the top with a pipet.

centrifuge shows that the bottom layers are rich in the heavy particles and lack the light particles, whereas the top layers show the opposite distribution.

Quite a different type of fractionation results from chromatography on columns of diethylaminoethyl cellulose (DEAE) [9, 10]. Extremely high resolution can be achieved giving a separation of various proteins as shown in figure 2. Nucleoprotein appears as a prominent peak in the elution diagram of the total cell juice but not in the diagram obtained with the 100,000g supernatant fluid (fig. 3). The corresponding ultraviolet diagrams show that there are in fact two nucleoprotein peaks: the first peak consists of nucleoprotein of high molecular weight which can be spun down in the centrifuge; the second

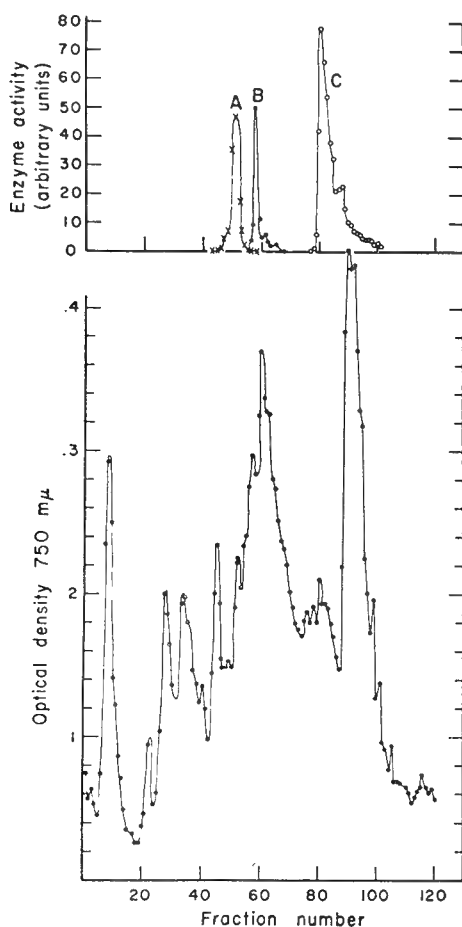


Fig. 2. Cell suspension washed and broken as described by Bolton et al. [8]; 0.5 g wet weight of cell juice adsorbed on DEAE column ($1\text{ cm}^2 \times 20\text{ cm}$) and eluted with concentration gradient 0 to 0.7 M of NaCl in tris-succinate buffer plus magnesium. Lower curve, total protein indicated by Folin reaction; upper curve, assay for activity of three different enzymes. One-milliliter samples collected in fraction collector.

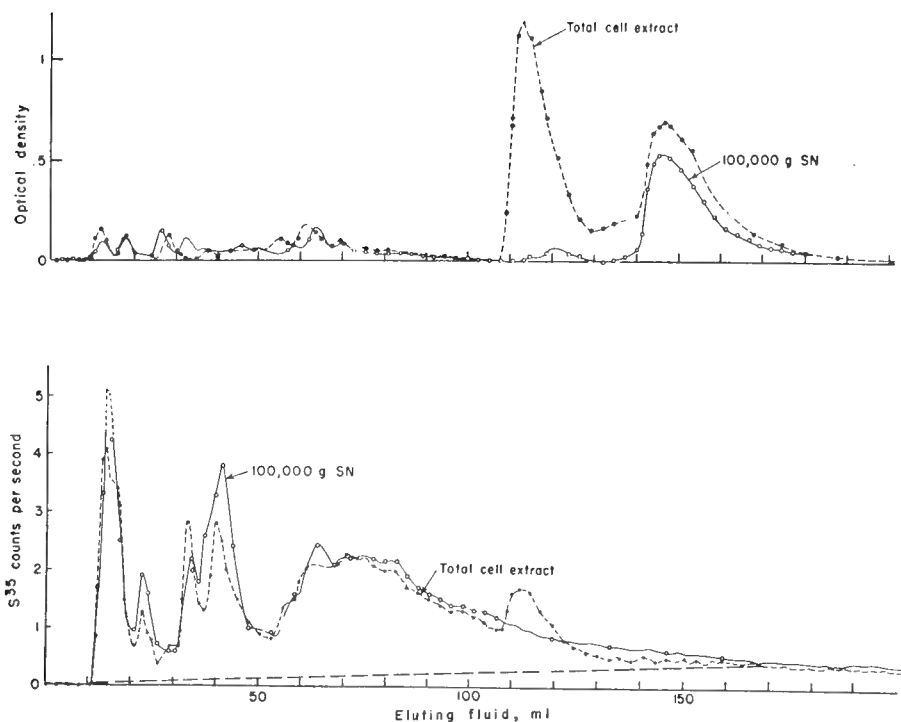


Fig. 3. Elution patterns of total cell juice and supernatant fluid of 100,000g 2-hour spin. Upper curve, optical density at 254, indicating nucleic acid concentration; lower curve, S^{35} radioactivity, indicating protein. Note nucleoprotein peak which is missing in 100,000g SN.

is partly nucleoprotein and partly due to free DNA and RNA which still remain in the 100,000g supernatant fluid.

The elution pattern is not sensitive to the size of the particles. The same pattern is obtained whether the microsome pellet is composed mostly of the large (80 S) particles or of the smaller 20 to 40 S particles that result from magnesium deficiency. Compare figures 4*a* and *b*.

Microsome pellets when resuspended and analyzed on the column show the nucleoprotein peak together with a quantity of other protein which depends on the method of preparation (fig. 4). A part of the contamination of the microsome pellet is due to small bits of cell wall, and another part is due to nonparticulate protein. In 2 hours at 100,000g, roughly 70 per cent of the β -galactosidase is sedimented. See also Dagley and Sykes [5, 11]. Accordingly the least-contaminated preparations of ribosomes are those obtained by resuspending a microsome pellet and centrifuging again in the swinging bucket head (fig. 4*c*).

Unfortunately the column cannot be used to prepare purified ribosomes because the material eluted from the column is quite different from that originally adsorbed. When the fractions containing the nucleoprotein peak are

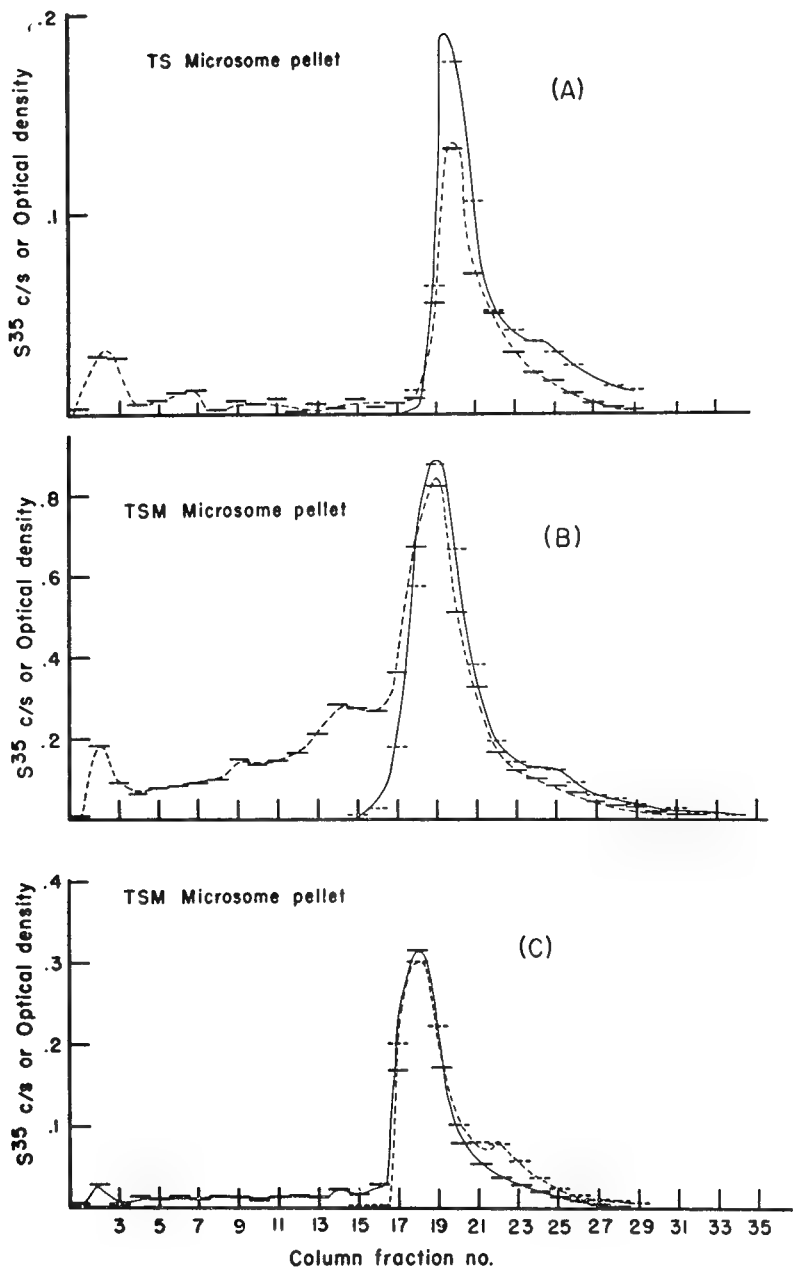


Fig. 4. Elution patterns of microsome pellets. *a*, 100,000*g* 2-hour pellet without magnesium present; *b*, same with magnesium present; *c*, microsome pellet resuspended and fractionated with swinging bucket centrifuge.

centrifuged (100,000g, 2 hours), a colorless glassy pellet is formed which contains approximately 65 per cent of the protein and nucleic acid. This pellet resuspends easily and completely. The analytical centrifuge shows that it contains peaks in the 20 to 40 S region, whereas the 80 S peak was most prominent in the original material. The ratio of nucleic acid to protein in this pellet (measured by optical density at 260 m μ and S³⁵) is twice that of the starting material, and the elution pattern obtained when the pellet is rerun on a DEAE column is very different (fig. 5).

These changes appear to be caused by the column material and not by the salt of the eluting fluid. Ribosomes exposed to molar NaCl show a reduction in size but no change in composition or elution pattern.

The fractionation and analysis procedures outlined above are beginning to yield some useful information about the composition and function of the ribosomes. The purified ribosomes are markedly different from the microsome pellet. For example, the microsome pellet contains considerable phospholipid (table 1); the ribosomes, little if any. Moreover, the nucleic acid to protein ratio is somewhat variable in the crude microsome pellet, but the purified ribosomes

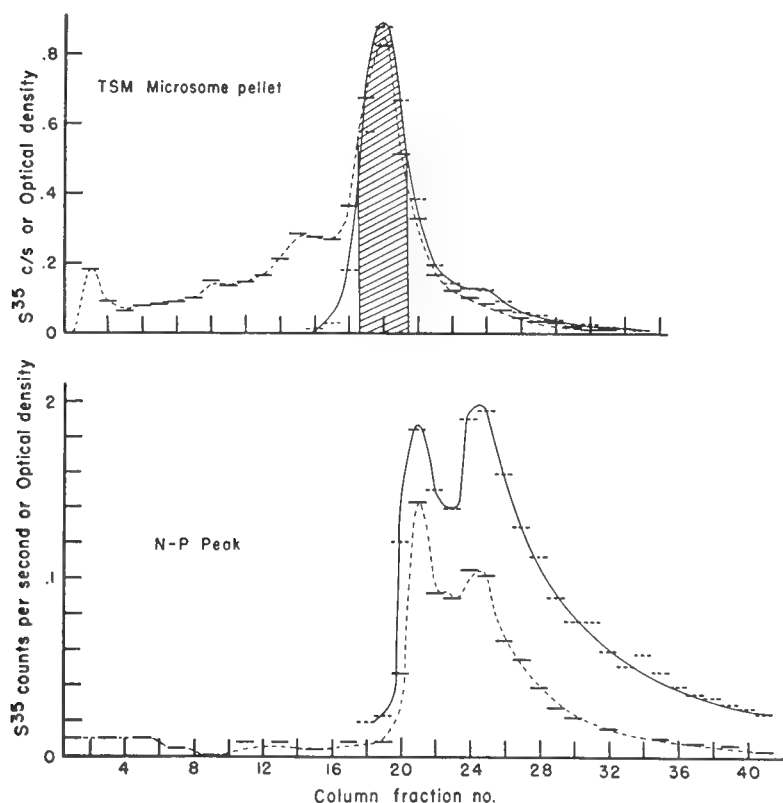


Fig. 5. Nucleoprotein peak of elution pattern spun down and rechromatographed. Note change to elution pattern like that of nucleic acid.

TABLE 1. Chemical Fractionation of *coli* Components

	Whole Cell	Cell Wall 30,000g pellet	Microsome 100,000g pellet	Soluble 100,000g SN
Small molecules	8	1	1	6
Lipids	7	4	3	0
RNA	15	0	13	2
DNA	3	0	0	3
Protein	67	15	13	38
	—	—	—	—
Total	100	20	30	50

obtained from the swinging bucket give a constant ratio indicating two amino acids per nucleotide (NA/P=60/40 measured by absorption at 260 mμ and Folin [12] test for protein).

The protein of ribosomes differs from other proteins of the cell. Purified ribosomes were obtained from cells grown with C¹⁴ glucose as the sole carbon source. The protein, after hydrolysis and chromatography, showed an amino acid distribution in which glutamic acid, alanine, glycine, and lysine were proportionately higher than in the whole cell, whereas methionine and aspartic acid were lower. In this protein neither cysteine nor cystine seems to be present.

The absence of cystine can best be shown by growing the cells in the presence of S³⁵O₄ to label cystine and methionine. After hydrolysis and chromatography the radioactivity of methionine and cystine can be measured. In the protein of the whole cell there is approximately twice as much methionine as cystine [13]. In ribosomes purified in the swinging bucket centrifuge this ratio is 10:1. In nucleoprotein eluted from the column and sedimented the ratio is greater than 100:1.

Alternatively the lack of cystine can be demonstrated without hydrolysis and chromatography. Cells containing S³⁵ cystine and S³² methionine were grown by adding S³⁵O₄ and S³² methionine to the medium. To prevent even a slight leakage of S³⁵ into methionine, a methionine-requiring mutant was used [13]. The sulfur radioactivity per unit protein of the nucleoprotein (obtained by column analysis of a microsome pellet and sedimentation of the nucleoprotein fraction of the eluate) was 50 times lower than that of the whole cell. Since the usual occurrence of cystine is only 1 per 60 residues, its occurrence in the nucleoprotein is less than 1 per 3000.

Kinetic studies of the fractions obtained from the column are also in progress. S³⁵ has been used to follow incorporation into protein. Exponentially growing cells were exposed to the tracer for varying periods of time and then broken and their constituents analyzed. The specific radioactivity of the protein fractions was measured by TCA-precipitable S³⁵ and Folin reaction color. When the cells are exposed to the tracer for a prolonged period (steady state) the specific radioactivity varies throughout the elution pattern by a factor of roughly 3, being lowest in the nucleoprotein fraction. These variations are simply due to variations in the sulfur content. Other cells were grown for three generations in

a nonradioactive medium after exposure to the tracer. In this treatment any intermediates which have a rapid turnover should lose their radioactivity. The resulting "persistent pattern" was entirely similar to the "steady-state pattern," and no protein components could be identified as intermediates.

Finally, cells were exposed to the tracer for short periods. After a 4-minute exposure the resulting "pulse pattern" was similar to the "steady-state pattern" except that the radioactivity of the nucleoprotein peak was only one-half of that expected from the "steady-state" pattern. A similar result was obtained with cells exposed for 4 minutes to a mixture of C^{14} -labeled amino acids.

Similar experiments carried out with $P^{32}O_4$ give much more striking results. Figure 6 shows the macromolecular region of the elution patterns obtained with cells exposed to the tracer for increasing periods of time. The radioactivity appears first in a quite distinct fraction of the elution pattern, passing through at a later time to the other regions. In the steady-state and persistent patterns the phosphorus radioactivity was proportional to the optical density (at 260 $m\mu$). Thus the DEAE column is capable of resolving the nucleic acid and nucleoprotein into fractions that seem to be precursors and products. Similar kinetic differences were also observed by Creaser, who used ECTEOLA columns [9] to analyze alcohol-extracted nucleic acid [14].

The analysis of these data runs into a number of complications. The leading peak is composed solely of RNP, but the secondary peak is an unresolved mixture of RNP, RNA, and DNA. Furthermore, the pool of low-molecular-weight precursors to RNA is large and may or may not be in equilibrium with the smaller pool of DNA precursors [14].

A rough analysis can be made on the basis of several simplifying assumptions. Assume first that the low-molecular-weight precursors of the macromole-

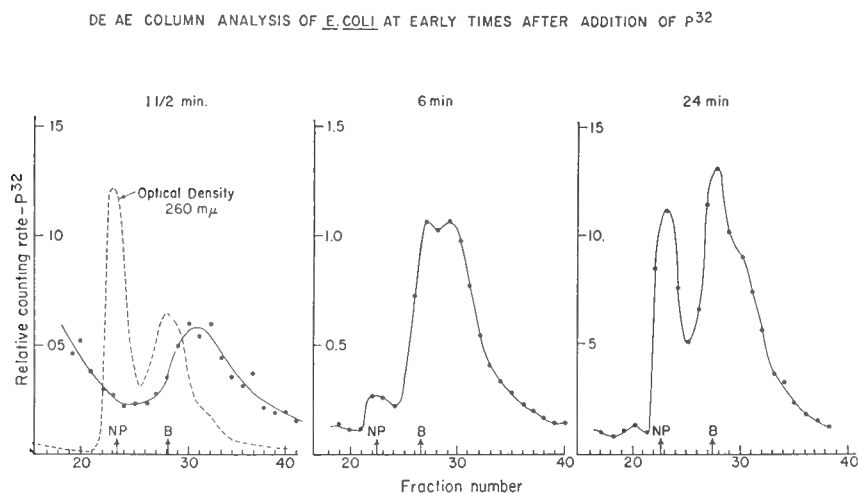


Fig. 6. Elution patterns of cell extracts after growing cells were exposed to $P^{32}O_4$ for times indicated. Only a small region of the elution pattern is shown.

cules have the average specific radioactivity of the TCA-soluble pool. Second, since the persistent and steady-state runs show all the macromolecular ultraviolet-absorbing material to be uniformly labeled, assume that it is the end product and that its specific radioactivity is that of the nucleoprotein. Analyzed on this basis the data point to an intermediate containing roughly 10 per cent of the nucleic acid.

Some other characteristics of this intermediate have been determined. Very short exposures to the tracer were used to prepare cell juices which were shown by column analysis to have most of the P^{32} in the intermediate and little in the end products. Most of the low-molecular-weight materials were removed by washing the cells with water before breaking. This material was analyzed in the swinging bucket centrifuge. The results (fig. 7) show that the TCA-precipitable radioactivity sediments at less than half the rate of the ultraviolet-absorbing material, which is mostly in 80 S ribosomes. Incubation with RNAase showed the usual rate of release of nucleotides.

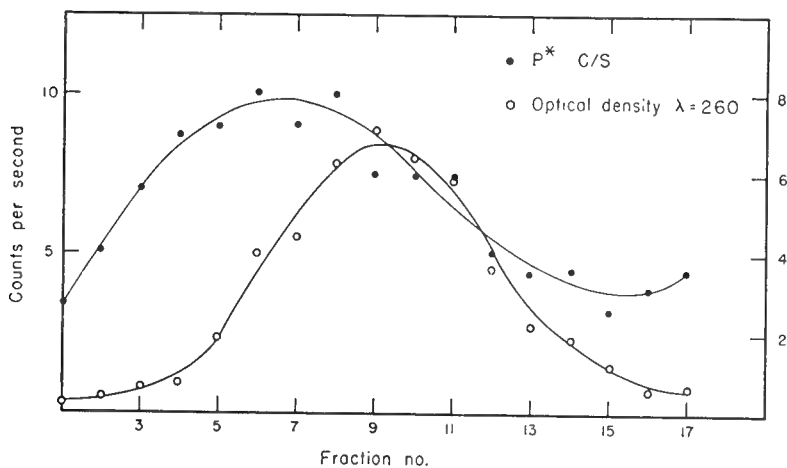


Fig. 7. Growing cells were exposed to P^{32} for 3 minutes, then broken, and the microsome pellet was analyzed in the swinging bucket centrifuge. Note that the maximum radioactivity does not correspond to the maximum of the ultraviolet absorption. Cf. figure 1.

Those findings, together with its column elution pattern, suggest that the intermediate is RNA of high molecular weight, either free or associated with less protein than the bulk of the nucleoprotein. It should be emphasized that neither lipids nor fragments of cell wall or cell membrane are eluted from the column, and it is observed that a large part of the P^{32} incorporated in short exposures is irreversibly bound to the column. An important part of the kinetics may thereby be missed.

DISCUSSION

To interpret the detailed workings of the cell in terms of its structural components, fractionation procedures are needed to separate those components. The

procedures outlined above are only a step toward the needed resolution, but they have already given indications that:

1. The 80 S ribosomes are composed of nucleoprotein of approximately two amino acids per nucleotide.

2. This composition is unaltered in the smaller disintegration products resulting from magnesium deficiency.

3. Adsorption followed by elution from the DEAE column causes disintegration into smaller particles of different composition containing approximately one amino acid per nucleotide.

4. The protein of the ribosomes is a special protein or at least a special class of proteins lacking cystine and cysteine. It is therefore doubtful that any of the enzymes that have been reported in the microsome pellet are actually in the ribosome fraction.

5. The protein of the ribosomes is most certainly not precursor to the non-particulate proteins. Such a relationship is ruled out by the data on the composition and on the kinetics of formation.

6. Incorporation of amino acids, sulfur, and phosphorus into nucleoprotein of the ribosomes shows a kinetic delay which indicates that the ribosomes have a macromolecular precursor.

7. This precursor has properties suggestive of nucleic acid or nucleoprotein of a low protein content.

These findings when checked and verified will be useful in providing further conditions that must be met by any theory of protein synthesis. The low initial specific radioactivity found in the ribosome fraction differs markedly from the high initial specific radioactivity found in the deoxycholate-insoluble part of the microsome fraction of rat liver [15]. A partial explanation for the difference may be that the nucleoprotein has been stripped clean of adhering newly formed protein by the column; it is not a complete explanation, however, because the nonparticulate protein of the microsome fraction did not have a high initial specific radioactivity. More likely, the difference arises from the difference in the growth rates. If the ribosomes furnish the templates for protein synthesis, and if chains of 150 amino acid residues are produced by the ribosomes, then each of the 10,000 ribosomes of a *coli* cell must produce one polypeptide chain per 10 seconds to give the observed rate of protein synthesis. If one polypeptide chain adheres to each ribosome, after 4 minutes' exposure to the tracer only 1/24 of the newly formed polypeptide chains would be found still adhering to the particles. To show kinetic effects in protein synthesis with these rapidly growing organisms it will be necessary to use much shorter exposures to the tracer.

The synthesis of the particles themselves appears to be a distinctly different process, as it proceeds at a more leisurely rate. Even after 24 minutes there are still marked departures from the steady-state distribution. These findings are compatible with, but certainly do not prove, the idea that the smaller particles observed in the cell juice are not simply bits broken off during disruption of

the cell, but that they have biological significance and that they may represent stages in the growth cycle of the particles.

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Comment. The early attempt at sedimentation analysis shown in figure 7 should be compared with the resolution obtained later (see III.B.2). The present paper, however, shows two classes of "rapidly labeled RNA." If a demonstration of "rapidly labeled RNA" is considered satisfactory proof of the messenger theory, this paper contains the proof, two years before the theory was published.

A lack of cystine in ribosomes was reported here. This was erroneous, and a correction was reported in Year Book 58: "Cystine content of ribosomes. Experiments reported last year showed very little cystine in the ribosomes. This result had considerable significance as it indicated that ribosomal protein could not be the source of the soluble proteins which contain cystine. On repeating this measurement (using the mutant strain of E. coli ML 304D), cystine appeared in the ribosomes. Another repetition with E. coli B again showed only traces of cystine. Further experiments then showed considerable cystine (cystine/methionine = 1/6) in ribosomes of E. coli B." Possibly cystine desulfurase was active during the prolonged procedures used to purify the ribosomes.

Richard B. Roberts.

III.C.3 Ribosome Synthesis during Unbalanced Growth

(Reprinted, by permission, from Biochemical and Biophysical Research Communications, vol. 1, no. 4, pp. 244-247, October 1959.) (Received September 28, 1959.)

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The thymineless mutant of *Escherichia coli* (15 T⁻) can synthesize ribonucleic acid (RNA) and protein even though the synthesis of deoxyribonucleic acid (DNA) is prevented by lack of thymine (Barner and Cohen, 1954; Hanawalt, 1958). We have used this mutant to determine whether the absence of DNA synthesis caused any alteration in the distribution of RNA among the soluble RNA and the different classes of ribonucleoprotein particles (ribosomes). Ultracentrifugation and column chromatography were used to separate the various types of RNA, and P³² labeling served to distinguish newly formed nucleic acid from that originally present.

Methods

A culture of *E. coli* 15 T⁻ growing exponentially in tris-glucose medium (Roberts et al., 1957) containing 3 μ g thymine/ml and 0.7 μ mol PO₄³⁻/ml was harvested, then washed with and resuspended in the tris-PO₄³⁻ medium lacking thymine. Glucose was added, and the culture was then aerated at 37° after being divided into two portions, one with and the other without thymine (3 μ g/ml). P³² was added immediately to observe the entire time course of incorporation or at appropriate times to observe the incorporation after different periods of incubation without thymine. Samples of the culture were taken for chemical fractionation (Roberts et al., 1957), for centrifugal fractionation and chromatography (Roberts, Britten, and Bolton, 1958), for measurement of bacterial mass (optical density at 650 m μ) and nucleic acid content (optical density at 260 m μ), and for plate counts. The nucleic acid content of the various fractions was measured by absorption at 260 m μ , and the protein content was measured by Lowry's modification of the Folin method (Lowry et al., 1951). The P³² of trichloroacetic acid (TCA) precipitable compounds was measured by adding TCA, filtering on membrane filters, and counting the material on the filter (Britten, Roberts, and French, 1955).

Results

In the control culture with thymine present the growth was exponential during the period of observation (2 hours), and there was no change in the relative proportions of the various components.

The culture lacking thymine exhibited these previously observed features (cf. Hanawalt, 1958). The growth (measured by OD at 650 m μ) was linear; RNA was synthesized at an approximately constant rate; protein and phospholipids were synthesized at increasing rates; the incorporation of P³² into DNA was drastically reduced; and the number of cells capable of forming colonies dropped by more than a factor of 10 after 60 minutes' incubation without thymine.

The characteristics of the RNA produced during this period of unbalanced growth were then examined. Three methods were used that had been shown to be capable of distinguishing the abnormal RNA produced in the presence of chloramphenicol (Pardee, Paigen, and Prestidge, 1957; Aronson and Spiegelman, 1958; Carnegie, 1958): (1) Samples of cells taken after 90 minutes' growth without thymine were disrupted and analyzed in the Spinco model E ultracentrifuge. More than half of the RNA of these cells had been formed during unbalanced growth. The pattern of particles (which showed ribosomes having sedimentation constants of approximately 20S, 30S, 50S, 70S, and 85S) was typical of those commonly observed in exponentially growing *E. coli* B. The control culture of 15 T⁻ growing with thymine showed a slightly smaller proportion of the 30S and 50S particles. (2) Samples were taken periodically from both cultures, and the cells were harvested and washed. The cells were broken, and the walls and membranes were removed by a short period of centrifugation. The ribosomes were then pelleted from the wall-free extracts by centrifugation in the angle head rotor of the Spinco model L centrifuge (2 hours, 40,000 rpm). 55 \pm 5 per cent of the ultraviolet-absorbing material was sedimented from eight samples taken (at 15-minute intervals) from the culture lacking thymine. The same proportion sedimented in four samples taken from the control culture. (3) Chromatography on DEAE-cellulose of a ribosome pellet obtained from cells that had grown 90 minutes without thymine gave an elution pattern typical of ribosome pellets obtained from *E. coli* B (cf. Roberts, Britten, and Bolton, 1958). Thus the RNA formed during unbalanced growth is initially distributed in the usual proportions among soluble RNA and the various classes of ribosomes.

As the total RNA increased linearly with time and the ribosomes made up a nearly constant proportion of the total, the ribosomes must also have been synthesized at a constant rate. Direct measurements of the nucleic acid content or the P³² of the ribosome pellets likewise indicated a constant rate of synthesis, but this technique was less accurate because of variations in the efficiency of breaking the cells.

In contrast the soluble RNA seems to be synthesized at an increasing rate. The increasing rate was most readily observed by measuring the incorporation of P³² into the soluble and ribosome fractions during 15-minute intervals after various periods of incubation without thymine. This technique showed a doubling of the rate of synthesis of soluble RNA after 75 minutes of incubation without thymine, whereas the rate of ribosome synthesis did not increase. As the newly formed soluble RNA contributes only 15-20 per cent of the total ultraviolet absorption, its increasing rate of synthesis cannot be demonstrated if only the total is measured.

Discussion

The RNA formed during the unbalanced growth caused by chloramphenicol is abnormal, as can be shown by its lower sedimentation rate, its changed ultracentrifuge pattern, and its chromatography. In contrast these techniques fail to show any abnormality in the RNA that is formed during the unbalanced growth of 15 T⁻ caused by the absence of thymine. Accordingly, the synthesis of ribosomes does not require the concurrent synthesis of DNA. The rate of ribosome synthesis, however, no longer increases after synthesis of DNA stops, suggesting that the quantity of DNA may be a rate-limiting factor in the synthesis of ribosomes.

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III.C.4 High-Resolution Density Gradient Sedimentation Analysis

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Abstract. The principle of stability for a sample layered in a density-gradient liquid column is discussed, and a method for separating ribonucleoprotein particles by means of sedimentation in the ultracentrifuge is described.

In the process of our studies of bacterial ribonucleoprotein particles (ribosomes), the need arose for a method of sedimentation analysis which would supply separated samples of the ribosomes of *Escherichia coli* (1-3). These particles have sedimentation constants of about 20, 30, 50, 70, and 100S. Adequate separation of these classes has been obtained by sedimentation at 105,000g through a density-gradient stabilized liquid column, use being made of the principle that the stabilizing density gradient must always exceed the inverted density gradient introduced by the sample. Since the method is mechanically simple and probably of general application, it is described separately in this report.

Density gradients are commonly applied to prevent mixing in liquid columns which are used for zone analysis by means of centrifugation or electrophoresis. However, this method has been limited to very small quantities of material, since the sample itself may introduce a region of density instability. So long as the density increases in the direction of the gravitational (or centrifugal) field, the gradient will exercise a stabilizing force against mixing which occurs as a result of mechanical disturbances or temperature gradients. If the density gradient is locally inverted due to the presence of a sample, the liquid containing the sample will stream through the less dense underlying layers. This process does not necessarily stop

when the stream reaches a region of equal density, since the stabilizing solute (usually more rapidly diffusing than the sample) will diffuse into the stream and may continually reestablish a condition of instability.

The sample layer shown in Fig. 1A is initially stable. However, as soon as the sample is moved downward (or the stabilizing solute diffuses into the sample layer), the inverted density gradient will cause streaming. The inverted density gradient may be avoided if the sample is introduced (Fig. 1B) with a concentration gradient opposite to that of the stabilizing gradient, provided the inverted density gradient due to the sample itself is significantly less than the stabilizing density gradient.

Usually a maximum quantity of sample can be analyzed when both gradients are linear. The amount of sample which can be handled rises with the square of the width of the sample layer, since it is the *gradient* in density and not the maximum density of the sample which determines stability.

The instability of sharply defined sample layers has been previously recognized and offset (4) by stirring the sample layer to reduce the inverted gradient at the lower edge of the band. Stable inverted sample gradients of complex shape have been created through the use of mixing chambers (5). However, the large sample capacity and the simplicity of the inverted linear sample gradient have not previously been mentioned in the literature.

This principle has been applied successfully both for analysis by electrophoresis and for analysis by sedimentation in the ultracentrifuge. Since the latter application has been of great

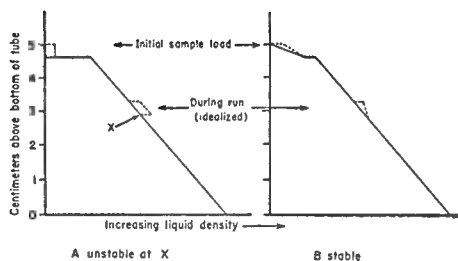


Fig. 1. Conditions for stability in a density gradient liquid column. Solid line, stabilizing gradient; dotted line, contribution due to a sample in process of analysis.

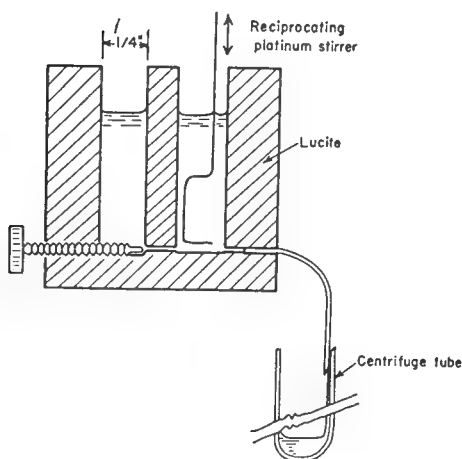


Fig. 2. Mixing chamber for the production of linear stabilizing gradients. The left chamber is filled with 2.4 ml of 5 percent sucrose, and the right, with 2.2 ml of 20-percent sucrose. After filling and after starting of the mixing motor, the center valve is opened and the exit tubing is turned down to touch the side of the centrifuge tube. The sucrose solution then runs down the wall of the tube. If the 4.6 ml are delivered in 10 to 15 minutes, the succeeding lighter solution floats on the underlying liquid with little mixing.

importance to our experiments on synthesis of (1, 2) and by (6) the ribosomes of *E. coli*, it is described here in detail.

A linear stabilizing density gradient of sucrose (20- to 5-percent) was prepared with a modification of the linear gradient mixing device of Bock and Ling (7), shown in Fig. 2. Such gradi-

ents are stable for many hours, and only moderate care need be taken in handling the tubes. The same device was then used to introduce the inverted sample gradient. For this purpose the left-hand chamber was loaded with 0.2 ml (for example) of buffer containing 5 mg of ribosomes per milliliter, and the right-hand chamber with 4-percent sucrose in the same buffer. The sudden step in sucrose concentration from 5 to 4 percent makes it possible to start the sample gradient without undue mixing. The stabilizing gradient can be reduced when the sample to be analyzed is small. Gradients of 10- to 3-percent sucrose have been used occasionally and are quite adequate.

This whole process was carried out in the cold room with solutions at 1° to 4°C. The tube was loaded into the precooled swinging bucket rotor (Spinco SW39) and centrifuged for the appropriate time. As quickly as possible at the end of the run, the tube was gently lifted out of the rotor and mounted in a device which perforated the bottom of the centrifuge tube with a hypodermic needle (ground to a short, double-sided point) located about 1 mm above the bottom of the tube. After removal of a piano wire which kept the hypodermic tubing clear and free of air bubbles, the contents of the tube were run out in 25 equal cuts by drop counting.

Figure 3 shows the result of use of this system for examination of the radioactivities of the smaller particles of *Escherichia coli* after a short period of incorporation of radioactive sulfate. A growing culture of *E. coli* was starved of sulfur for 30 minutes, and then $S^{35}O_4^{--}$ was added. After 1 minute, carrier $S^{32}O_4^{--}$, S^{32} -cystine, and S^{32} -methionine were added to displace the radioactivity of the rapidly-turning-over soluble proteins synthesized by the ribosomes (6). Fifteen seconds later the culture was suddenly chilled to 0°C, the cells were washed and broken, and the ribosomes were harvested in the preparative ultracentrifuge. The ribosome pellet was resuspended in appropriate buffer, and a sample was loaded in an inverted linear gradient layer, as

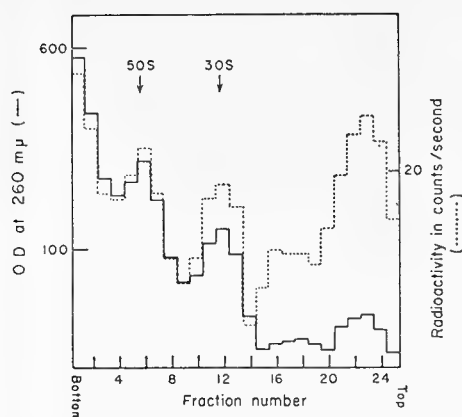


Fig. 3. Example of the use of density gradient stabilized sedimentation analysis for measurement of radioactivity incorporated into the smaller ribosomes during 1-minute exposure to $S^{35}O_4^{2-}$. Solid line, optical density at 260 $m\mu$ of 0.2-ml samples diluted to 1.2 ml. Dotted line, trichloroacetic acid precipitable radioactivity.

described above. The tube was then centrifuged (37,000 rev/min, for 150 minutes) to pellet most of the 70 and 100S ribosomes in order to spread out the 20-to-50S region for a close examination of the smaller ribosomes.

The peaks in the diagram correspond closely with the peaks shown by the analytical centrifuge which was used to determine the sedimentation constants.

A series of experiments of this type (2) shows that the newly synthesized ribosomal protein and ribonucleic acid appear first in the smaller ribosomes (20, 30, 50S) and later in the larger ones (70, 100S).

A sample of the width discussed above has provided the best compromise between high resolution and a useful quantity for analysis, although the resolution could probably be improved to the limit set by diffusion if smaller samples and great care were used. For the example shown in Fig. 3, the full

width ($\frac{1}{2}$ max.) due to diffusion alone would be about 1 mm or about $\frac{1}{8}$ of the observed width. Since the width is due to causes other than diffusion, equivalent resolution could be obtained for objects very much smaller than the ribosomes. For example, serum globulin (molecular weight, 170,000, about 7S) would require about 12 hours of centrifugation, and the resulting diffusion width would be about 3 mm. For the preparation of large quantities of material, very broad sample gradients are useful. In the preparation of pure 30S particles from a mixture of 30 and 50S ribosomes, 2.4 ml of a very concentrated ribosome suspension are placed in the left chamber, and 2.4 ml of 20-percent sucrose, in the right chamber. In this way 10 to 20 mg of pure 30S particles may be obtained in a single run.

It is worth noting that, while the centrifugal force doubles from the top to the bottom of the tube in the swinging bucket rotor, the viscosity of 20-percent sucrose is about twice that of water. When correction is made for the density of the sucrose solution, it is found that the sedimentation velocity of ribosomes is very nearly constant throughout the length of the centrifuge tube.

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31 July 1959

Comment. Improvements to this technique which increase the resolution appreciably are described in III.B.2. More colorful illustrations of the method and a description of its application to the analysis of polysomes appear in an article by A. Rich in the *Scientific American*, **209**, 44-54, 1963. Richard B. Roberts.

III.C.5 Variations in Bacterial Ribosomes

(Reprinted, by permission, from Biochimica et Biophysica Acta, vol. 39, pp. 563-564, 1960.)

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Ribosomal particles are an important constituent of all bacterial cells. Particular attention has been focussed on them recently due to their role in protein synthesis, a clear demonstration of this function in bacteria having been made by McQUILLEN, ROBERTS AND BRITTEN¹. Study of a large number of ultracentrifugal analyses indicates that the ribosome content of a bacterial cell is characteristic of the physiological state of the cell. The absolute quantity of ribosomes in a cell varies according to the medium in which they are growing, being greater when the cells are growing more rapidly. Furthermore, radical changes in the relative proportions of the various components are discernible as the cell passes from the growing to the resting state and *vice versa*. The ribosomes of resting cells, which have exhausted the supply of the energy source, glucose, are almost entirely of one class whereas exponentially growing cells show peaks corresponding to at least four classes of ribosomes.

A culture of resting cells was prepared by growing *Escherichia coli* B overnight in a glucose (0.8 mg/ml)-salts medium under forced aeration at 37°. In this way the glucose supply had been exhausted some 12 h prior to the commencement of the experiment. After the addition of more glucose, 100-ml samples were taken at intervals and poured upon crushed frozen growth medium. The cells were then harvested and washed three times in 0.01 *M* tris(hydroxymethyl)aminomethane, 0.004 *M* succinic acid and 0.01 *M* magnesium acetate buffer, pH 7.6. Breakage of the cells was effected by means of a modified French pressure cell at 10,000 lbs./in.². The resulting cell juices were examined in a Spinco Model E ultracentrifuge equipped with Schlieren optics. Sedimentation coefficients were estimated from the plates and corrected for the viscosity change of water to 20°. Fig. 1 shows the progressive changes in the particle pattern during the first 15 min.

The addition of glucose caused appreciable conversion of the large single component into smaller particles during the first 1.5 min. Calculation of the sedimentation coefficients showed that the new particles appearing were the well known ones of 70, 51 and 32 *S*^{2,3}. The quantity of the largest component continued to decrease until by 11 min the pattern resembled that of a typical exponential culture. Furthermore, the sedimentation coefficient of the fastest moving component was now 86 *S* and none of the original 100 *S* component remained.

The onset of renewed growth could not be adequately assessed from turbidity measurements but study of the ³⁵SO₄⁼ uptake showed that no appreciable protein synthesis occurred during the first 7 min (Fig. 2). The period between 7 and 11 min corresponded to that of an increasing rate of protein synthesis. At the end of this

time changes in the ultracentrifuge pattern were almost complete. Thus the recommencement of growth seems to be correlated with the establishment of the normal ratios between the amounts of the various classes of ribosomes.

A similar study of the reverse transition into the resting state suggested that changes in the particle pattern are equally rapid in this direction. Although there is more uncertainty in assessing the time at which the last traces of glucose are swept up by the cells, the ultracentrifugal analysis of two samples taken 5 min apart, just as the turbidity of the culture reached a plateau, gave two very different patterns.

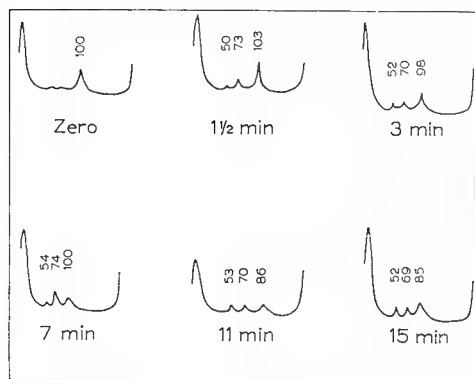


Fig. 1. Sedimentation patterns of cell juice extracted at various times after the addition of glucose. Photographs after 430 sec at 50,740 rev./min with bar angle 30° .

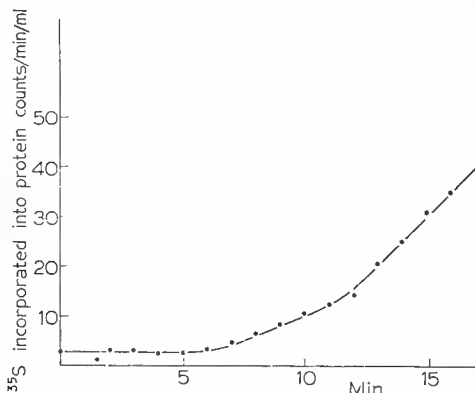


Fig. 2. Uptake of ^{35}S into fraction insoluble in trichloroacetic acid by cells recovering from glucose starvation. ^{35}S and glucose added at time zero.

While the first was typical of an exponential culture, the second had no components of 85 S but consisted mainly of 100 S ribosomes.

It is the predominance of the two larger particles of approximately 85 and 70 S which characterises exponentially growing cells. Ribosomes with an S number as high as 100 appear exclusively in extracts prepared from resting cells. These extracts contain only traces of the slower components. The most important process taking place in the lag before growth of the bacterial cell can recommence may be this fundamental reorganization of the ribosomal material.

The exact relationship between the three large particles having sedimentation coefficients of 70, 85 and 100 S is not known. It seems possible, however, that the three objects represent different physical states of the same particle since they may be readily interconverted *in vitro* by changing the Mg^{++} concentration³. An analogous transformation may be brought about by an *in vivo* mechanism of the cell. Accordingly 100-S ribosomes may be regarded as inert ribonucleoprotein which can rapidly be converted into protein-synthesising machinery.

¹ K. MCQUILLEN, R. B. ROBERTS AND R. J. BRITTEN, *Proc. Natl. Acad. Sci. U.S.A.*, 45 (1959) 1437.

² A. TISSIÈRES AND J. D. WATSON, *Nature*, 182 (1958) 778.

³ Report of the Biophysics Section, Department of Terrestrial Magnetism, Carnegie Inst. Wash. Year Book, 1959, p. 58.

III.C.6 Sedimentation Characteristics of Bacterial Ribonucleoprotein Obtained at Different Periods during the Cell-Division Cycle

(Reprinted, by permission, from *Biochimica et Biophysica Acta*, vol. 39, pp. 150-151, 1960.) (Received October 3, 1959.)

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A large proportion of the ribonucleic acid of bacteria occurs in the form of ribonucleoprotein particles¹. In growing *Escherichia coli*, these particles (ribosomes) are distributed among a number of different classes having sedimentation coefficients of approximately 20 S, 30 S, 50 S, 70 S and 100 S. The sedimentation coefficient of the largest particle varies between 70 S and 100 S depending on the magnesium concentration. At low concentration (10^{-4} M) the 70-S and 100-S particles, dissociate into 30-S and 50-S particles^{2,3}. The 30-S and 50-S particles are observed, however, even when the magnesium concentration is high enough to prevent this dissociation. They appear to be the precursors of the larger particles as they are the first to incorporate radioactive materials⁴. More recently evidence has been obtained that the larger ribosomes provide the principal sites for protein synthesis⁵.

Using unsynchronized cultures it is not possible to tell whether the different groups of particles exist at all times in each cell or whether the particles change from one class to another in synchrony with the division cycle of an individual cell. If the second alternative were correct samples taken from a synchronized culture at different times during the division cycle should show marked changes in the proportions of the different particles.

Preliminary experiments showed that the ribosomes derived from an exponentially growing culture of *Alkaligenes fecalis* gave a distribution pattern similar to that obtained from *E. coli*. Also the same dissociation occurred at low magnesium concentration. Accordingly the synchronized cultures of *Alk. fecalis* which were available seemed quite suitable for observation of the ribosome pattern during synchronized division.

A 380-ml synchronized culture of *Alk. fecalis* was obtained by a modification of the filtration method described previously⁶. Synchrony was measured by following viable-colony count and total bacterial-cell count, the latter measured in the Coulter Particle Counter (current setting of 6, threshold 12 maximum gain) on samples taken at intervals and fixed in 0.2 % formalin. The growth pattern of this culture is shown in Fig. 1. 50-ml samples were taken as indicated by arrows 1-7 and dumped onto 20 g of crushed frozen medium at approximately -70° . The samples were then centrifuged at 2° and washed twice in buffer containing 0.01 M tris(hydroxymethyl)amino-methane, 0.004 M sodium succinate, 0.01 M magnesium acetate pH 7.6. The samples were finally suspended in 0.6 ml of the same buffer and frozen until they could be analyzed. For analysis, the frozen samples were thawed and broken in a modified French pressure cell and immediately run in the model E Spinco ultracentrifuge.

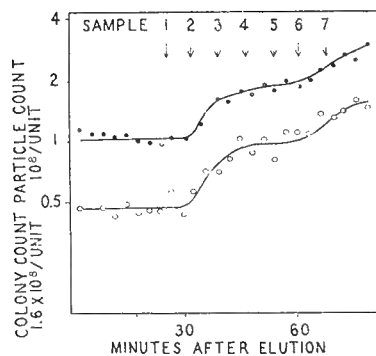


Fig. 1. Synchronous growth of *Alk. fecalis* LB. Bacteria eluted from filter paper used for selection of large cells at zero time. Arrows indicate time of removal of samples for RNA analysis. ○, colony count; ●, particle count.

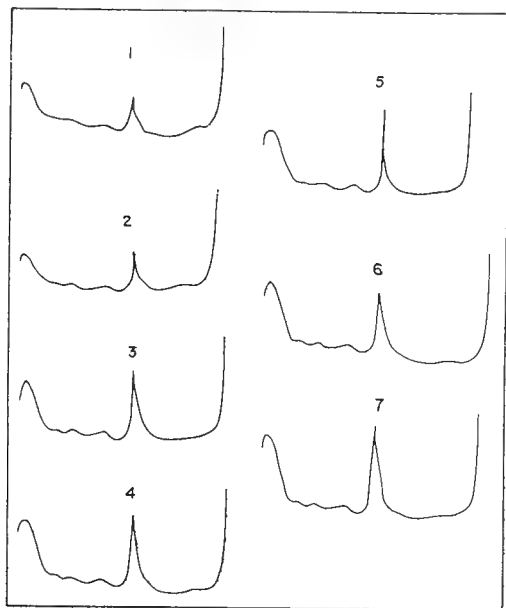


Fig. 2. Analytical ultracentrifuge patterns of *Alk. fecalis*. Samples were taken from a synchronized culture at the times indicated in Fig. 1. The sedimentation coefficient of the largest component is approximately 70 S.

The results are presented in Fig. 2. As may be seen, all of the components were present in the extracts made at different times from the synchronized culture. At no time in the division cycle was one form of ribosome present in quantities strikingly different from those found in exponential cultures. From this it can be concluded that the ribosome pattern which has been studied in exponential cultures represents that of the individual cell and is not merely an average pattern resulting from the summation of different patterns present in cells at different phases of the division cycle.

¹ H. K. SCHACHMAN, A. B. PARDEE AND R. Y. STANIER, *Arch. Biochem. Biophys.*, 38 (1952) 245.

² E. T. BOLTON, B. H. HOYER AND D. B. RITTER, in R. B. ROBERTS, *Microsomal Particles and Protein Synthesis*, Pergamon Press, N.Y. 1958.

³ A. TISSIÈRES AND J. D. WATSON, *Nature*, 182 (1958) 778.

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⁵ K. MAQUILLEN, R. B. ROBERTS AND R. J. BRITTEN, *Proc. Natl. Acad. Sci. U.S.*, 1959, in the press.

⁶ K. G. LARK, *Can. J. Microbiol.*, 4 (1958) 179.

III.C.7 Structure of Ribosomes from *Escherichia coli* as Revealed by Their Disintegration

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Ribonucleoprotein (RNP) particles are found in living cells and are known to be vitally involved in the synthesis of protein molecules. Although the intact particles have been repeatedly observed in the electron microscope (Hall & Slayter, 1959; Huxley & Zubay, 1960) virtually nothing is known about their internal organization. In the hope of discovering something of their architecture we have followed with the electron microscope the breakdown of RNP particles from *E. coli*.

RNP particles were obtained from exponentially growing cultures of *E. coli* as described by Bolton, Britten, Cowie, McCarthy, McQuillen & Roberts (1959). The particles were suspended in a buffer (TSM) containing 0.01 M-tris, 0.02 M-succinic acid, 0.01 M-magnesium acetate, with pH adjusted to 7.6. Under these conditions the RNP particles are known to be aggregated in the form which has a sedimentation coefficient of 100 s (Tissières, Watson, Schlessinger & Hollingworth, 1959; Britten & McCarthy, 1959).

Breakdown of the particles was effected by removal of magnesium. Using the method of Elson (1959) it was shown that when this is done the RNA is made acid-soluble, presumably by the now activated RNase enzyme known to be present in latent form in the intact particle (Elson, 1959; Bolton & McCarthy, 1959). Figure 1 shows the progress of the reaction for a 0.1% suspension of RNP particles in 0.1 M-EDTA (ethylenediaminetetra-acetic acid) at pH 7.6. It is seen that the reaction is largely completed in two hours. During this period $90 \pm 10\%$ of the RNA is rendered acid-soluble.

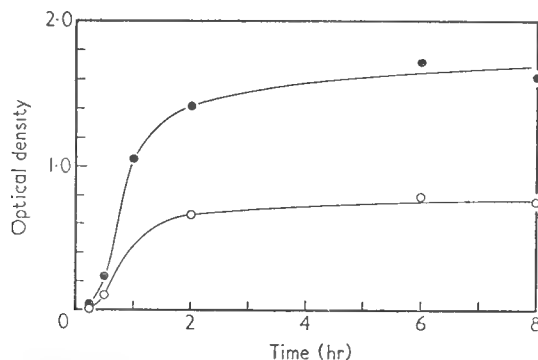


FIG. 1. Rate of production of acid-soluble RNA from 0.1% suspension of RNP particles in 0.1 M-EDTA, at pH 7.6. ● 260 mμ; ○ 280 mμ.

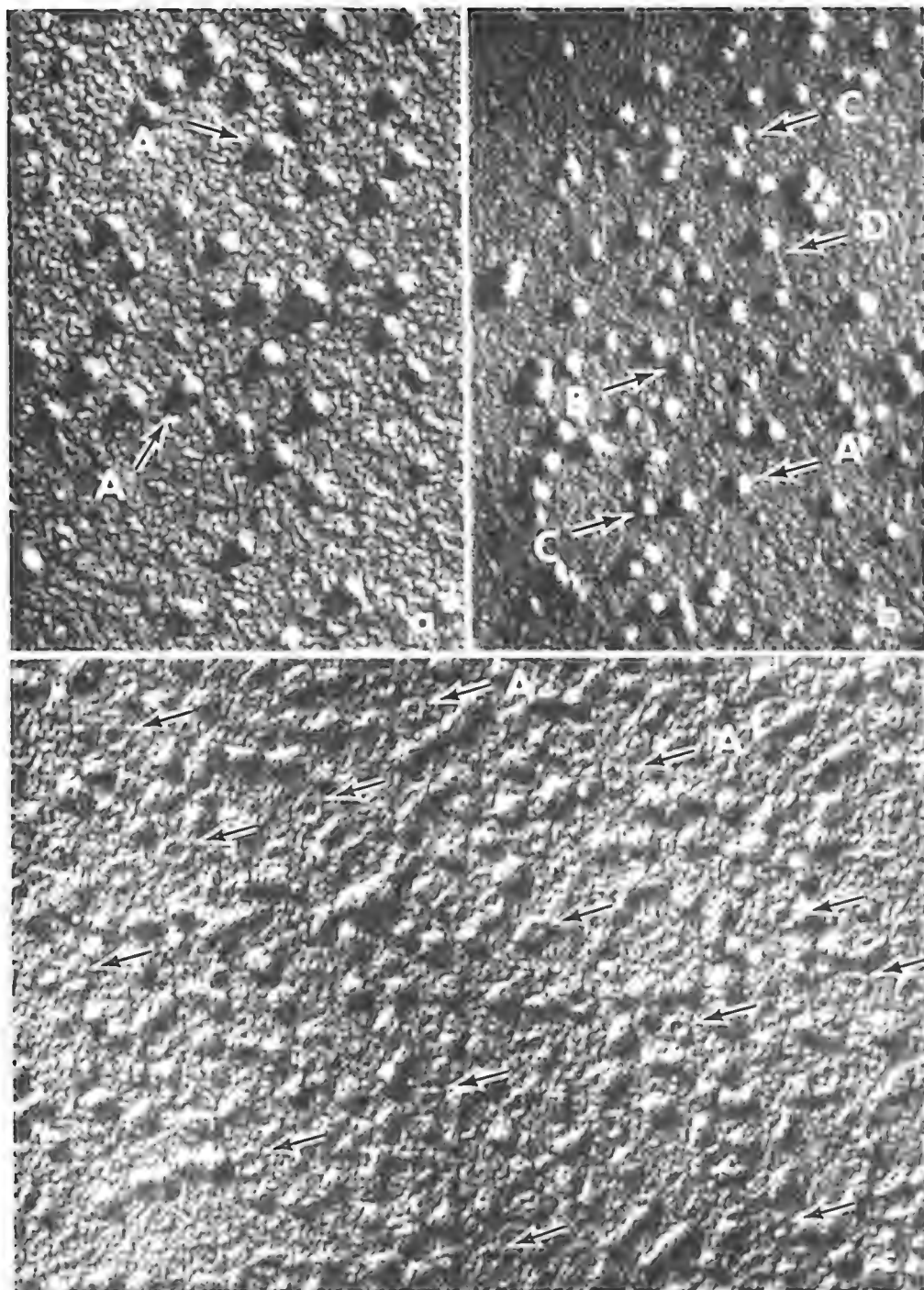


PLATE I(a). Electron micrograph of RNP particles after short dialysis from TSM buffer into distilled water. Most of the particles are the 70 s doublets. The components of the doublets are readily seen at A. Magnification $\times 120,000$.

(b) Electron micrograph of RNP particles after long dialysis from TSM buffer into distilled water. 50 s particles are visible at A and 30 s particles at B. Fibrils being released by the 50 s components can be seen at C. The 50 s and 30 s particles slightly separated and joined by a fibril are shown at D. Magnification $\times 120,000$.

(c) Electron micrograph of RNP particles after dialysis from TSM buffer into EDTA followed by distilled water. The hollow cup-like structures shown by the arrows are believed to be the protein shells which remain after the RNA has become digested. At A the structure has been loosened considerably and the globular components are readily visible. Magnification $\times 120,000$.

For electron microscopy single drops (about 0.1 ml.) of 5×10^{-4} % suspensions of RNP particles in TSM were placed on formvar films which floated on 50 ml. of water or 0.1 M-EDTA solution at pH 7.6. In some experiments EDTA was added to the suspension of RNP particles. The liquid beneath the film was periodically pipetted out and replaced. At the end of the dialysis, the films with the drops were picked up on copper grids, air-dried, shadowed with platinum at an angle of 4 to 1 and examined in the electron microscope. Varying degrees of degradation were obtained by stopping dialysis after different lengths of time.

After 2 hr of dialysis against each of 3 changes of distilled water the majority of particles appear elongated and consist of 2 portions. Their long and short dimensions are approximately 250 Å and 180 Å. The two components are clearly visible at A in Plate I(a). The doublet character observed here is consistent with that of the 70 s particle which consists of one 30 s and one 50 s. The dimensions given by Hall & Slayter (1959) for this are 200 by 170 Å. The coarse background at this stage of dialysis indicates that all the salt has not yet been removed. Thus after this dialysis the 100 s particles have broken down to 70 s particles but not further.

After 24 hr of dialysis with 4 changes of distilled water the early stages of disintegration of the particles are visible. The doublet 70 s components have separated into the rather spherical 50 s component (A in Plate I(b)) and the more asymmetric 30 s component (B in Plate I(b)). This separation is to be expected on removal of magnesium. In addition many of the particles have short fibrils attached to them (C in Plate I(b)). The thickness of these fibrils is very roughly 30 Å. They presumably represent material released from the particles. This fibrillar material which is seen emerging from the particles may well be the RNA loosened by removal of the magnesium ions. The 30 Å estimated diameter of these fibrils is too great for single-stranded RNA and probably even for double-stranded RNA. Conceivably these strands also carry attached protein.

Occasionally one sees the 50 s and 30 s particles a small distance from each other but joined by the fibril (D in Plate I(b)). This suggests that in some of the 70 s particles, the 30 s member is so tightly bound to the fibrillar component of the 50 s particle that the fibril is released from the 50 s particle before the 30 s breaks away. It is interesting in this connection that Tissières, Schlessinger & Gros (1960) have described a fraction of the 70 s particle which is relatively resistant to breakdown on removal of magnesium. This fraction is responsible for essentially all the *in vitro* incorporation of amino acids.

More complete removal of magnesium was effected by dialysing against EDTA for 3 hr, then twice against water for 2 hr. Adding EDTA to the suspension and dialysing it away after 2 hr led to similar results. Plate I(c) shows a typical area of the specimen. The structures observed here were similar to those found after very long dialysis against water. A large number of cup-like hollow structures are visible. They are indicated by arrows in Plate I(c). Their diameter is somewhat variable between 200 Å and 400 Å. The central hollow has a diameter varying from "hardly visible" to about 200 Å. The structures with larger external diameters also have larger hollows suggesting that some of the structures are loosened more than others. The size and shape of these structures compare with those of the 50 s particles suggesting that they are derived from them.

RNP particles are known to consist of about 60% RNA and 40% protein. Our chemical evidence cited earlier indicates that the breakdown involves a removal and degradation of essentially all the RNA component. The hollow cup-shaped structures which remain after degradation must then be the protein components. The view that the RNA of the RNP particles is embedded in protein is consistent with the observations of Elson (1959) and of Tissières *et al.* (1959) that the particles are relatively resistant to RNase.

The walls of the cup-shaped structures are not smooth. Particularly where the degradation has been extensive and the structure is loosened (A in Plate I(c)), it is possible to discern components of the wall which are globular and which have a diameter about 20 to 50 Å.

After degradation of the particles, our micrographs showed no structures which could be clearly related to the 30 s particles. Experiments using purified particles are now in progress to discover their fate.

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III.C.8 Studies of *E. coli* Ribosomal RNA and Its Degradation Products

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ABSTRACT The RNA of *E. coli* ribosomes has been extracted by the phenol method. The 70S ribosomes contain RNA molecules of 28 and 18S almost exclusively. When the 70S ribosomes are dissociated to 30 and 50S ribosomes the former contain only the 18S RNA and the latter a mixture of 28 and 18S RNA. There are also present, however, small quantities of ribosomal RNA having sedimentation coefficients of between 4 and 8S. These small molecules are particularly abundant in the smaller ribosomes present in the cell extract and account for most of the RNA of 20S ribosomes.

In addition it has proved possible to degrade the large molecules of RNA to a series of smaller molecules. Removal of magnesium ions from the growing cell, extensive dialysis of the RNA against a buffer of low ionic strength, and heating all resulted in such degradation. Three degradation products were observed having sedimentation coefficients of about 13.1S, 8.8S, and 4.4S. The integral sedimentation distributions of these preparations suggest a high degree of homogeneity among the molecules of each of the three classes. The three sizes seem to result from sequential breaks in the molecules since the proportion of smaller molecules increases with time of treatment.

The molecular weights of the 8.8S and 4.4S molecules have been estimated as $144,000 \pm 4,900$ and $29,200 \pm 1,200$ respectively by the Archibald method.

INTRODUCTION

The evidence that ribonucleoprotein particles have a major role in protein synthesis is now rather extensive. In growing *E. coli* it has been shown that most of the cell protein is transiently associated with the ribosomes before appearing free as soluble protein (McQuillen, Roberts, and Britten, 1959). In *E. coli* about 80 per cent of the RNA exists as part of the ribosomal particles. The particles themselves are distributed among five size classes having sedimentation constants of 20S, 30S, 50S, 70S, and 85 to 100S. In exponentially growing cells the fourth class is the most abundant and may account for as much as 80 per cent of the total ribosome material.

Phenol extraction of the ribosomes enables one to obtain the RNA essentially

free of protein (Schramm and Gierer, 1956). The RNA consists mainly of two components with sedimentation coefficients of 15 to 18S and 23 to 28S (Timasheff, Brown, Colter, and Davies, 1958, Littauer and Eisenberg, 1959, Hall and Doty, 1959). However, most of these preparations of ribosomal RNA used for physical studies were derived from a mixture of particles in which the 70S particles were predominant. In the present study the RNA of each of the five particle groups was examined so as to be able to detect which components were present in each particle group. This paper presents these results together with a study of the degradation of the ribosomal RNA to material of lower molecular weight. In a following paper the kinetic relationships among the different RNA fractions are discussed in conjunction with the known relationships among the ribosome groups.

MATERIALS AND METHODS

E. coli ML 30 was grown in C medium (Roberts *et al.*, 1955) with glucose or maltose. An overnight culture was diluted with fresh C medium and grown to about 10^9 bacteria/ml. Cells were aerated at 37°C until the late exponential phase had been reached. All cells were harvested, washed twice in the cold with 0.01 M tris, 0.004 M succinic acid, 0.01 M magnesium acetate, pH 7.4 (TSM 10^{-2}). The cells were resuspended in a small volume and an extract prepared by passing them through the orifice of a French pressure cell at 10,000 to 15,000 psi.

Ribosome Purification

Purified samples of 70S ribosomes and their constituent 50S and 30S parts, and of the small quantities of 50S, 30S, and 20S ribosomes "native" to the cell extract may be obtained by centrifugal techniques.

70S. The cell extract was spun in the model L Spinco ultracentrifuge No. 40 rotor at 40,000 R.P.M. for 5 minutes. The pellet (40K 5 P)¹ was discarded and the supernatant centrifuged at 40,000 R.P.M. for 45 minutes. The pellet (40K 45 P) was resuspended in TSM 10^{-2} buffer and following a short spin in the Servall SSI (5 minutes) to remove aggregates, was recentrifuged 40K 45 minutes. The final pellet was resuspended in TSM 10^{-2} , examined in the ultracentrifuge for purity, and stored at -20°C. Such a preparation consisted mostly of 70S ribosomes since they are the most abundant. Further purification steps yielded pure 30S and 50S derived from the 70S.

50S. For the preparation of a purified 50S fraction the first 40K 45 minute pellet was resuspended in tris, 0.01 M, succinic acid, 0.004 M, magnesium acetate, 10^{-4} M, pH 7.4 (TSM 10^{-4}) and centrifuged at 40,000 R.P.M. for 180 minutes. The 70S breaks down to 50S and 30S in the low magnesium concentration. This pellet was resuspended in the same buffer and contained only 50S and 30S ribosomes mostly derived from the 70S. The 50S particles were purified by four successive centrifugations of 40K 90 minutes (Tissières *et al.*, 1959), resuspending the pellet each time in TSM 10^{-4} .

30S. Either of two procedures was employed to prepare pure samples of 30S ribosomes. The first involved layering the 50S/30S mixture on a sucrose gradient (Britten and Roberts, 1959) and centrifuging in the swinging bucket rotor at 37,000 R.P.M. for 150 minutes (SWB 37K 150). Fractions of about 0.2 ml each were collected and

¹ K, 1000 R.P.M.; P, pellet.

the absorption at 260 $m\mu$ determined. The region containing the 30S fraction was then dialyzed briefly in the cold against TSM 10^{-4} and pelleted by centrifuging in the swinging bucket rotor at 37,000 R.P.M. for 6 hours.

Alternately, the 50S/30S mixture was centrifuged SWB 37K 90 minutes. The top third was removed with a syringe and the 30S particles pelleted by centrifugation SWB 37K 6 hours. By either of these procedures purities in the range 90 to 95 per cent 30S ribosomes were obtained as routine.

Native 50S, 30S, and 20S. The quantities of 50S, 30S, and 20S available in a cell extract are very limited. The 20S ribosomes are especially elusive due both to their small size and to the fact that less than 5 per cent of the ribosome material is present in this form. The first 40K 45 minute centrifugation removed the bulk of the 70S ribosomes. The pellet resulting from a long centrifugation (40K 300 minutes) of the supernatant contained the remainder of the 70S and most of the 50, 30, and 20S ribosomes. It was resuspended in TSM 10^{-2} and after a brief centrifugation to remove aggregates it was submitted to sucrose gradient centrifugation. A spin of 37K 150 minutes was sufficient to pellet most of the remaining 70S ribosomes and resolve the 50S, 30S, and 20S. The appropriate fractions were collected and the various ribosomes collected by centrifugation.

Preparation of RNA

The phenol used for RNA extraction was first purified by shaking at room temperature with an equal volume of 0.02 M phosphate buffer, pH 7.0. In special cases the buffer also contained 10^{-8} M versene. After removing the aqueous phase, the treatment was repeated four or five times. Such phenol is adequate for preparing biologically active poliomyelitis RNA (Hoyer, 1960). Phenol purified by steam distillation or by means of a column of aluminum oxide (Tissières, 1959) was also used and parallel preparations of RNA using the two phenol samples were indistinguishable in their stability and sedimentation properties. The purified phenol was stored in the dark at 2°C.

The RNA was extracted by raising the magnesium concentration of the particle preparation to 10^{-2} M and adding an equal volume of the purified phenol. The mixture was sucked up and down with a syringe several times at room temperature and the phases then separated by centrifugation. The aqueous layer was removed and treated with phenol twice again. The final preparation was shaken five or six times with ether saturated with phosphate buffer to remove phenol, and nitrogen was bubbled through to remove the ether. Any insoluble material remaining was then removed by low speed centrifugation. The recovery was usually 60 to 70 per cent and protein contamination as measured by an S^{35} label was less than 0.1 per cent. The RNA preparations are identified as RNA₇₀, RNA₅₀, RNA₃₀, and RNA₂₀, being derived from the 70, 50, 30, and 20S ribosomes respectively. All preparations were frozen to -20°C in the deep freeze and thawed immediately before use.

Dialysis. All dialysis was carried out at 2°C with constant stirring. Visking dialysis tubing was washed in versene (0.01 M) solution and boiled in NaHCO₃ before use.

Apparatus

Sedimentation coefficients were measured in a model E Spinco ultracentrifuge equipped with schlieren and ultraviolet absorption optics. A Spinco analytrol was used to read the films and the arithmetic mean sedimentation coefficient determined by measuring the rate at which the midpoint of the concentration distribution sedimented. Alternatively the in-

tegral sedimentation distribution of the preparation was obtained by measuring the rate of sedimentation of a range of different percentages of the molecules from zero to 100 per cent (Kurland, 1960). The sedimentation coefficients were corrected for density and viscosity of water to 20°C.

RESULTS

Since examination of preparations in the ultracentrifuge was to be the primary criterion of the size of the RNA molecules, an extensive study was made of the dependence of the sedimentation properties of RNA upon the concentration of RNA

TABLE I
THE EFFECT OF CONCENTRATION AND IONIC
STRENGTH ON THE SEDIMENTATION COEFFICIENTS
OF RIBOSOMAL RNA

RNA source	Salts added to standard TS buffer (0.01 M tris 0.004 M succinic pH 7.4)	Concentration <i>mg/ml</i>	<i>s</i> _{20,w}
70S	None	2.7	13.0
			17.3
70S	0.01 M KCl	2.5	12.8
			15.2
70S	0.05 M KCl	2.6	15.1
			18.1
70S	0.2 M KCl	2.5	14.9
			18.9
70S	0.4 M KCl	2.5	16.2
			22.3
70S	0.2 M KCl + 0.01 M magnesium acetate	2.5	19.7
			28.0
70S	0.4 M KCl + 0.01 M magnesium acetate	2.5	20.6
			28.1
70S	0.01 M magnesium acetate	2.7	20.6
			27.1
70S	0.01 M magnesium acetate	2.7	20.8
			27.1
70S	0.01 M magnesium acetate	0.056	19.8
			28.6
50S	0.01 M magnesium acetate	2.5	18.9
			26.4
50S	0.01 M magnesium acetate	0.060	20.0
			28.8
30S	0.01 M magnesium acetate	2.4	18.2
30S	0.01 M magnesium acetate	0.052	19.2
20S*	0.01 M magnesium acetate	1.8	4.50
			(17.6)
20S‡	0.1 M NaCl	0.060	4.40
			(19.7)
			(24.8)

* See Fig. 1. ‡ See Fig. 2.

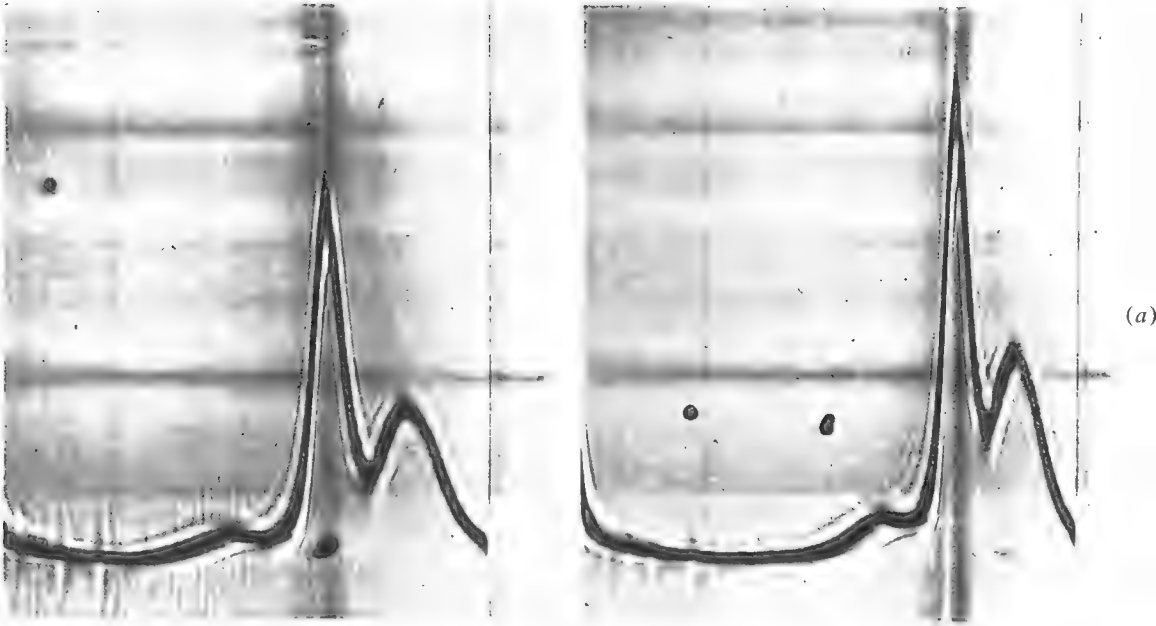


FIGURE 1 (a) Purified 20S ribosomes in TSM 10^{-2} . Pictures at 10 minutes (right) and 14 minutes (left) after reaching 50, 740 R.P.M. Concentration 2.1 mg/ml.

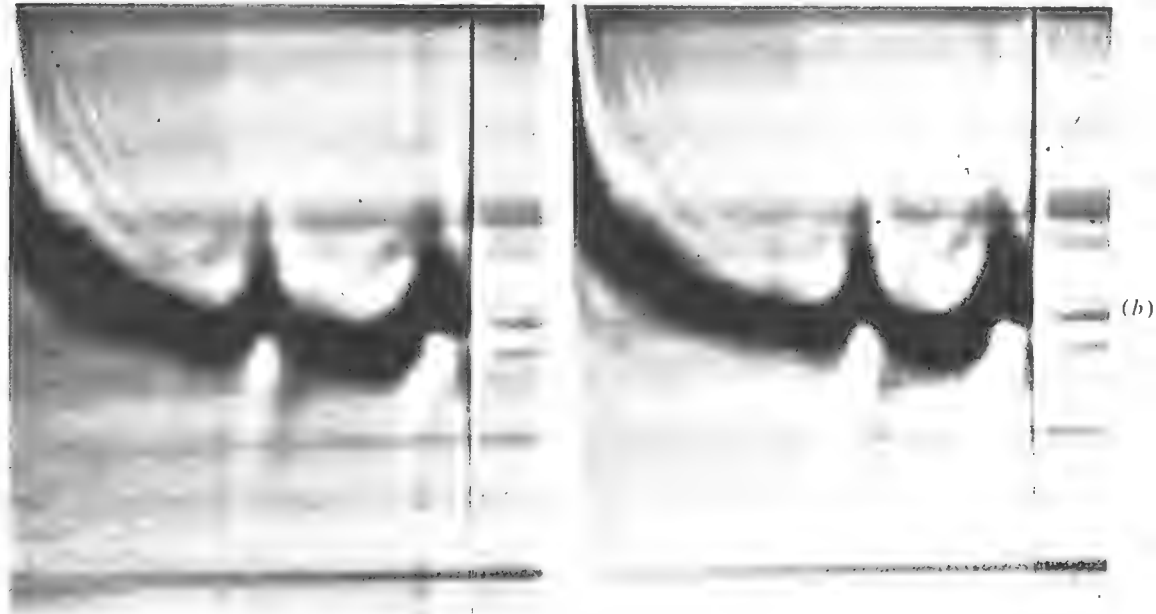


FIGURE 1 (b) RNA extracted with phenol from preparation shown in (a). Pictures at 19 minutes (right) and 27 minutes (left) after reaching 50, 740 R.P.M. Concentration 0.7 mg/ml. (The convection "spikes" in these figures are considered to be minor because of results of other centrifuge runs.)

and the composition of the solvent. At concentrations in the region of 2 mg/ml there was a considerable effect of ionic strength on the sedimentation coefficients of the RNA preparations. In 0.01 M tris buffer the sedimentation coefficients of the RNA from 70S particles were 13.0 and 17.3 (Table I). The addition of KCl from 0.01 M to 0.4 M increased these values progressively up to 16.2 and 22.3. The addition of 0.01 M magnesium acetate to the buffer in the presence or absence of a high concentration of KCl made a further increase in the sedimentation coefficients to about 20 and 27. There is a marked effect of ionic strength on the viscosity of RNA (Kawade, 1959, Cox and Littauer, 1960) and these changes may reflect an alteration in the shape of the molecules. It is apparent then that magnesium ions cause a tighter coiling of the RNA molecules than do potassium ions. The dependence of sedimentation coefficient on RNA concentration is not marked, particularly in the presence of magnesium ions (Table I).

The sedimentation analysis of RNA₇₀ is summarized in Table I. For the RNA₅₀ of 50S particles derived from the 70S, the two components have approximately the same $s_{20, w}$ values as those in RNA₇₀ (Table I). The relative proportion of the two peaks did vary somewhat among different preparations. Thus the larger component could account for from about 25 to 75 per cent of the total. Changes in the magnesium or other cation concentration of the solution had no effect on the relative proportion of the two peaks. When a preparation was reextracted with phenol, however, a shift in the relative proportion of the two peaks was observed so that some of the larger component was apparently converted to the smaller. This shift could be due to removal of a protein component or to an alteration in hydrogen bonding.

There was only one major component in RNA₃₀ of 30S particles derived from the 70S. Measured $s_{20, w}$ values are given in Table I. The small amount of faster moving material often observed is probably due to contaminating 50S particles in the original preparation. It did not prove possible to produce a second faster moving component by changing the cation concentrations. Fig. 1 *a* shows a preparation of 20S particles. The slower moving material of about 10S is evidently contaminating protein. Chromatography on a DEAE-cellulose column shows that about half of the material is protein and the other half nucleoprotein (Roberts *et al.*, 1960). These authors have analyzed pure 20S ribosomes and shown them to be of similar composition to the larger ribosomes; *i.e.*, 60 per cent RNA and 40 per cent protein. On the basis of this and a sedimentation coefficient very close to 20 the molecular weight of the smallest ribosome would be about half that of the 30S; *i.e.*, 5×10^5 . The RNA of a 20S particle would therefore be expected to have a molecular weight of 3×10^5 and a sedimentation coefficient of about 13S. The faster moving of the two peaks has an $s_{20, w}$ of 17.6 (Table I). This large component could represent a dimer of a 13S molecule that is very readily formed upon extraction of the RNA in the presence of 10^{-2} M magnesium. Alternatively it could have resulted from contaminating larger particles but there seems to be more than could be attributed

to the small contamination visible in Fig. 1 *a*. The other component is 4.5S indicating another state of the RNA in these particles. Here again, the rather large amount found suggests that contamination is unlikely. An analysis of a second preparation of RNA from 20S particles at lower concentration using ultraviolet optics (Fig. 2) suggested that most of the RNA is about 4 to 5S, with a smaller quantity of larger molecules perhaps due to contamination of the preparation by traces of 30S and 50S ribosomes.

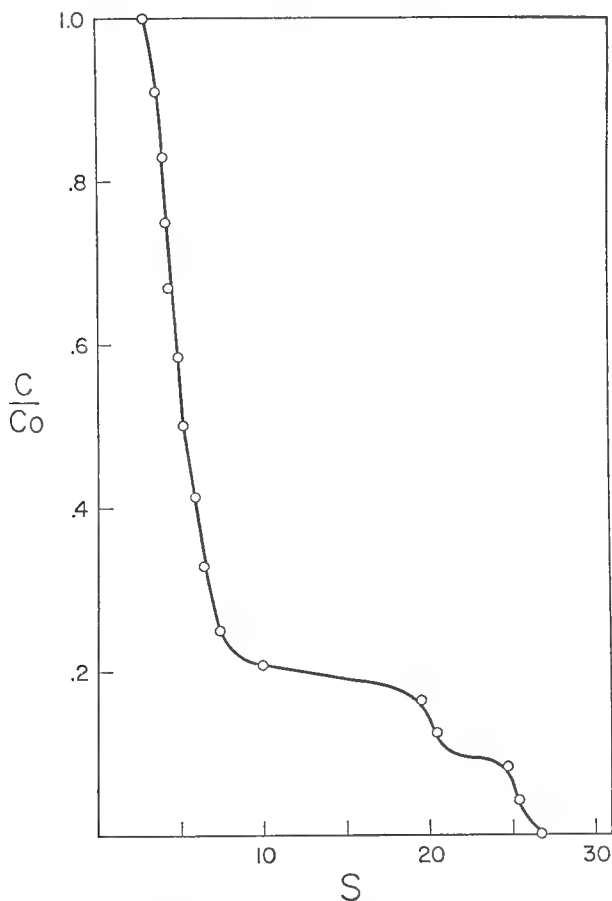


FIGURE 2 The integral sedimentation distribution of RNA from 20S ribosomes. Solvent 0.01 M tris, 0.004 M succinic acid, pH 7.4, 0.1 M NaCl. (Points are corrected for radial dilution.)

RNA Degradation

Instability of the RNA preparation with consequent breakdown into material of lower molecular weight is a characteristic feature of many of the studies made on both viral (Cheo, Briesen, and Sinsheimer, 1959, Boedtker, 1959) and ribosomal

(Hall and Doty, 1959, Kawade, 1959) RNA. Much of this instability has been previously ascribed to traces of nucleases in the preparation. This is, of course, a real possibility especially in the case of ribosomal RNA since the ribonucleoprotein itself contains a latent ribonuclease as an intimate part of the structure (Elson, 1958). In spite of this the RNA prepared as described showed no evidence of

TABLE II
THE DEGRADATION OF RIBOSOMAL RNA
TO SMALLER MOLECULES

No.	RNA source	Treatment	Solvent	Concentration <i>mg/ml</i>	$s_{20,w}$
1	70S	20 hrs. dialysis against TS	TS 0.2 M KCl	2.3	5.23 10.8 16.0 21.7
2*	70S	24 hrs. dialysis against TS	TS 0.1 M NaCl	0.05	4.40 9.00 13.15
3†	70S	32 hrs. dialysis against TS	TS 0.1 M NaCl	0.05	8.80
4	70S	70 hrs. dialysis against TS	TS 0.2 M KCl	2.1	4.28
5*	70S	72 hrs. dialysis against TS	TS 0.1 M KCl	0.05	4.15
6	50S + 30S	48 hrs. dialysis against 0.02 M PO ₄ , pH 7.2	TS 0.1 M NaCl	2.6	4.35
7	50S	68 hrs. dialysis against 0.01 M tris, 0.08 M KCl, pH 7.14	0.01 M tris, 0.08 M KCl, pH 7.4	1.9	4.68
8*	70S	Heated 90°C for 7 min in TS 0.2 M NaCl	TS, 0.2 M NaCl	0.05	4.40
9	Extract from cells Mg- starved 16 hrs	None	0.02 M PO ₄ , pH 7.4	2.9	4.14 10.9
10	Extract from cells Mg- starved 20 hrs	None	TS 10 ⁻² M Mg acetate	2.4	4.24 11.1
11*	Extract from cells Mg- starved 28 hrs	None	TS, 0.1 M NaCl	0.05	4.35

TS = 0.01 M tris, 0.004 M succinic acid, pH 7.4.

* The integral sedimentation distributions of preparations 2, 5, 8, and 11 given in Figs. 4*e, f, c*, and *b* respectively.

† The molecular weight of this preparation was determined by the Archibald method (1947). See Table III.

ribonuclease activity as measured by the appearance of acid-soluble P^{32} from labeled RNA, even when treated with 4.5 M urea for 48 hours to release any latent enzyme. This is in agreement with the results of Littauer and Eisenberg (1959).

It became obvious that degradation to lower molecular weights could be brought about by three separate procedures. These are described in order followed by a discussion of the molecular weights of the fragments produced.

Magnesium Removal. Extraction of RNA from ribosomes by phenol in the presence of 0.002 M versene produced components of sedimentation constants lower than 28 and 18S. The sizes of these components and their relative quantities were somewhat variable but the preparation of Fig. 3 is a typical one. The drop in the baseline of Fig. 3 *b* is an artifact of the analysis. Such results suggested that the presence of magnesium ions during RNA extraction might have a considerable influence on the stability of the RNA. Removal of magnesium from both the intact growing cells and from the RNA preparation was studied.

An investigation was made of the RNA extracted from magnesium-starved cells. These cells deprived of magnesium for at least 16 hours in a medium complete except for magnesium suffer breakdown of their ribosomes into the constituent RNA and protein moieties without losing any of the RNA by degradation to nucleotides (McCarthy, 1959).

The starved cells were disrupted in the pressure cell and the resulting cell extract poured immediately into ice cold phenol. After extraction in the usual manner the RNA was examined in the ultracentrifuge. There was often a faster moving peak of about 11S which tended to flatten out on continued centrifugation suggesting heterogeneity (Table II). The slower peak of about 4.2S did, however, appear to be reasonably homogeneous. Longer starvation periods reduced the relative amount of the faster component until most of the material was converted to a single component of 4.35S (Fig. 4 *b*). The degradation process appeared to reach a limit with the conversion of all the RNA to this molecular size. Of course, soluble RNA is extracted under these conditions and accounts for some 20 per cent of the total but the presence of ribosomal RNA in this mixture of 4 to 5S components can be demonstrated by chromatography on DEAE-cellulose (McCarthy and Aronson, 1961).

Dialysis. Dialysis of the RNA preparation against the standard tris buffer with 0.01 M magnesium acetate (TSM 10^{-2}) had little effect on the sedimentation coefficients of RNA for periods up to several days (Figs. 4 *a*, 4 *d*). If, however, the dialyzing medium consisted of tris buffer pH 7.4 without any added salt, the RNA was progressively degraded to a number of discrete smaller components (Figs. 4 *e*, 4 *f*). The relative amounts of these smaller components varied among different preparations. The rate of breakdown was diminished when the ionic strength of the dialyzing buffer was increased by the addition of 0.1 M KCl. With more extensive dialysis (48 to 72 hours) there was degradation to a limiting component of

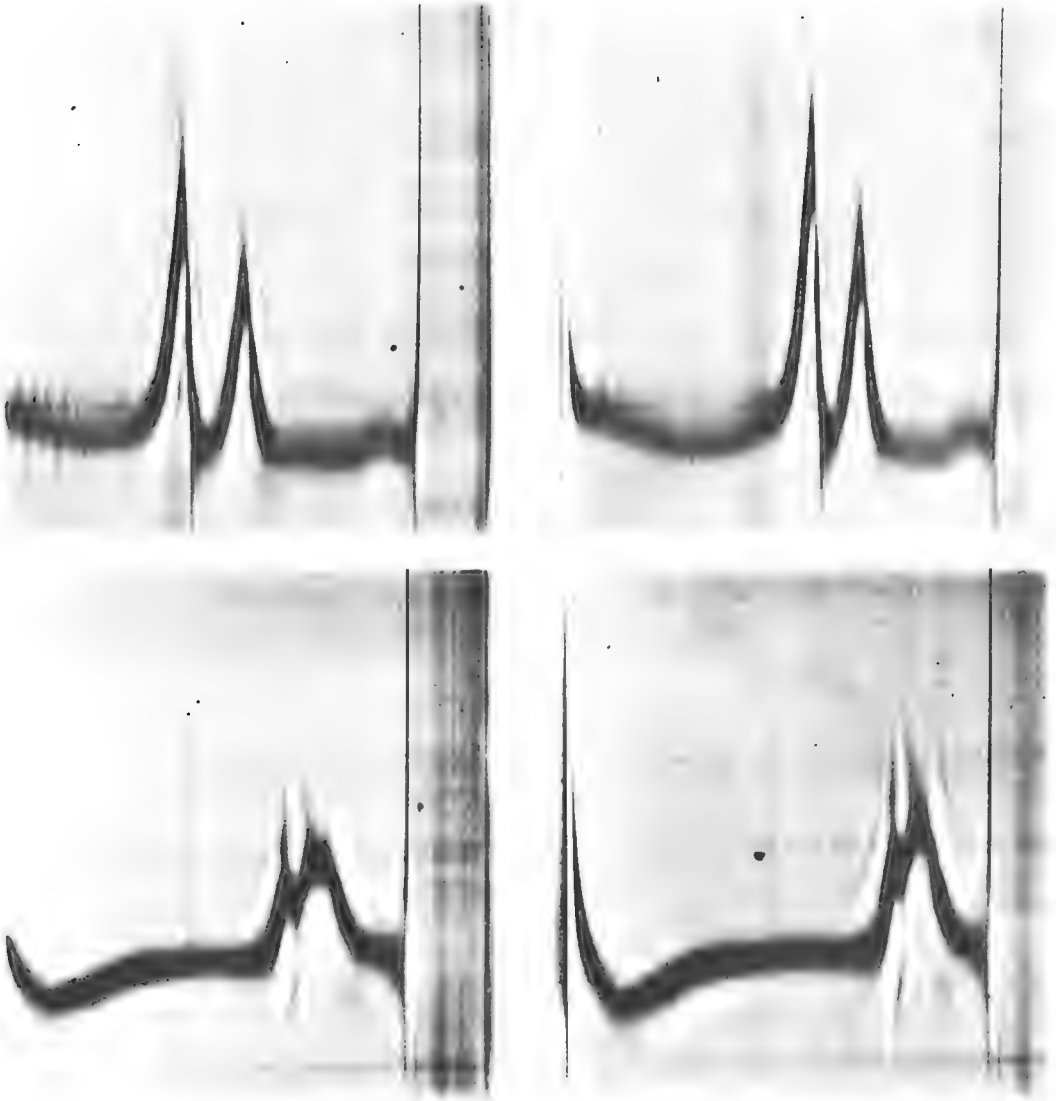


FIGURE 3 Two frames from schlieren pictures taken 14 minutes (right) and 18 minutes (left) after reaching 50,740 R.P.M. Sedimentation from right to left. Concentration 20.0 mg/ml. (a) RNA extracted from 70S ribosomes suspended in TSM 10^{-2} . Phenol-saturated with 0.02 M phosphate buffer, pH 7.3. (b) As (a) but phenol-saturated with 0.02 M phosphate buffer containing 2×10^{-3} M versene.

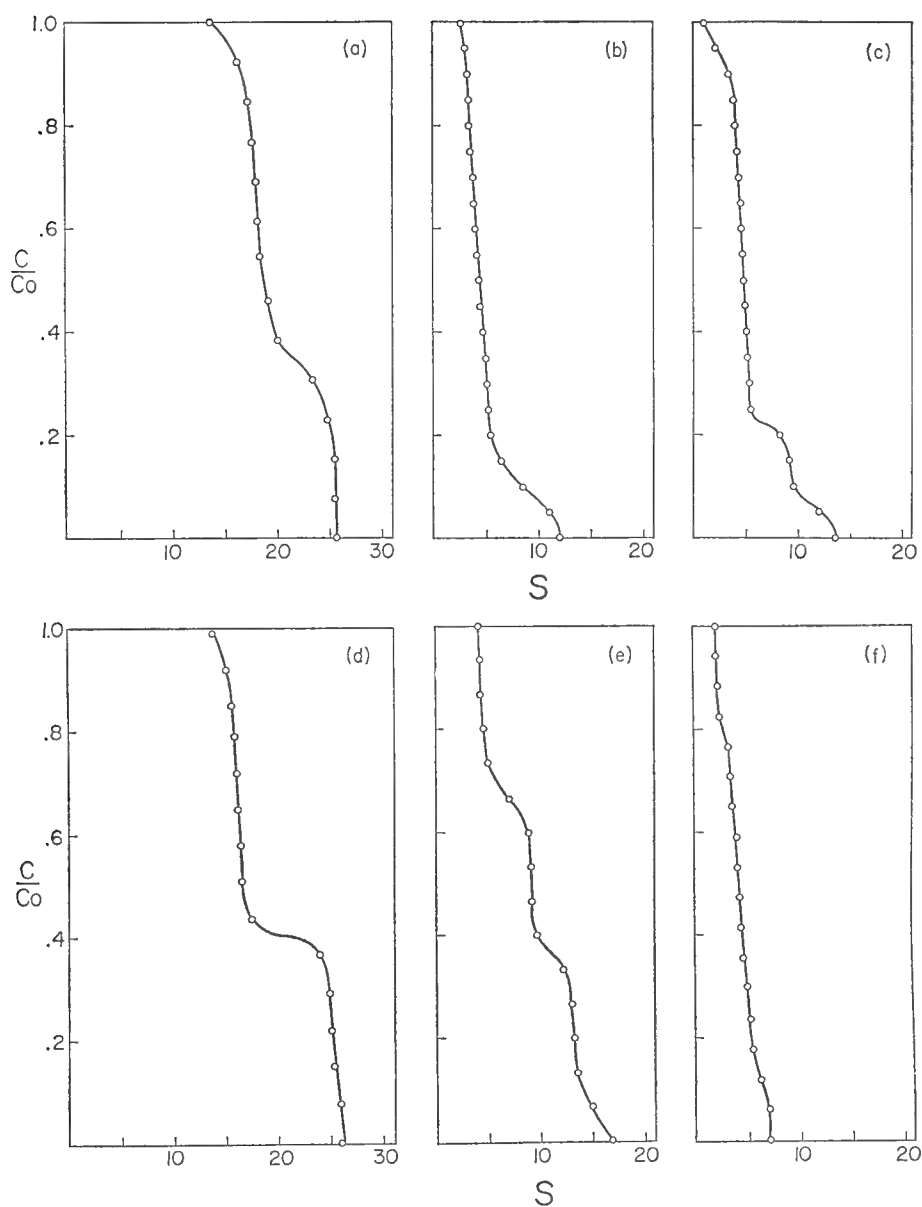


FIGURE 4 The integral sedimentation distributions of six RNA preparations. (a) RNA from 70S particles in $TSM 10^{-2}$. (b) RNA from magnesium-starved bacteria in TS, 0.1 M NaCl, pH 7.4. (c) RNA from 70S particles heated 90° 7 minutes in TS, 0.2 M NaCl, pH 7.4. (d) RNA of (a) dialyzed 60 hours against $TSM 10^{-2}$. (e) RNA of (a) dialyzed against TS 10^{-4} M, 32 hours, run in TS, 0.1 M NaCl, pH 7.4. (f) RNA of (a) dialyzed against $TSM 10^{-4}$ 72 hours, run in TS, 0.1 M NaCl, pH 7.4. (Points are corrected for radial dilution.)

TABLE III
MOLECULAR WEIGHTS OF TWO RNA PREPARATIONS

	Concentration <i>mg/ml</i>	R.P.M.	Time <i>min.</i>	Molecular weight	Average
1. 28 and 18S RNA from 70S particles dialyzed 2 days in TS, run in TS 0.1 M NaCl, $s_{20,w} = 8.80$	4.2	15,220	6 10 14 18 22 30	150,000 154,000 133,000 141,000 144,000 136,000	144,000 \pm 4,900
2. 28S and 18S RNA from 70S particles dialyzed 3 days in TS, run in TS 0.1 M NaCl, $s_{20,w} = 4.20$	2.9	24,630	6 10 14 18 22 26 30	29,700 27,000 28,600 29,900 30,100 29,100 28,800	29,200 \pm 1,200

4 to 5S (Fig. 4 *f*). Further dialysis did not produce smaller components, decrease the quantity of the 4 to 5S material, or cause any loss of dialyzable material to the outside buffer.

Heating. Periods of heating of a solution of RNA in tris buffer and 0.1 M KCl or 0.01 M magnesium acetate at 85°C for 10 or 15 minutes in the manner of Hall and Doty (1959) resulted in degradation to a mixture of smaller components between 4 and 18S. Heating at a higher temperature (90°C for 7 minutes) gave a limiting product of 4 to 5S (Fig. 4 *c* and Table III). Degradation of RNA to a limiting size molecule of 4S by heating has also been shown by Takanami (1959) for rat liver and Osawa (1960) for yeast ribosomal RNA's.

Molecular Weights

The integral sedimentation plots of the degraded components examined by ultra-violet absorption optics suggest a non-random degradation. There appear to be two distinct molecular species between the original 28 and 18S and the limiting 4 to 5S components (Fig. 4 *e*). The sedimentation coefficients fall in the range 8 to 9S and 12 to 13S. An attempt was made to isolate each of the three components for a molecular weight measurement. The 4 to 5S species was prepared by extensive dialysis until all the material was in this form. By following the course of the degradation process it proved possible to obtain the intermediate size molecule as a reasonably homogeneous preparation of 8.8S (Table II). Attempts to prepare the largest degraded molecule were not successful; preparations in the early stages of

dialysis always contained at least two components and were not suitable for molecular weight studies.

The molecular weights were estimated by the method of Archibald (1947) using the modification of Ehrenberg (1957). Table III shows the molecular weight values obtained from the top meniscus after different times of centrifugation. A partial specific volume of 0.550 was used. There was no marked trend in the values as a function of time, indicating reasonable homogeneity. The average molecular weights obtained were $144,000 \pm 4,900$ for the 8.8S component and $29,200 \pm 1,200$ for the 4.2S component.

DISCUSSION

It has been established that most of the ribosomal RNA can be accounted for as two components here described as 18 and 28S with molecular weights of 5.5×10^5 and 1.1×10^6 respectively (Kurlund, 1960). In addition Fig. 2 shows that smaller molecules can be obtained from the 20S particles. Further evidence for the occurrence of small RNA molecules in ribosomes is given in the subsequent communication (McCarthy and Aronson, 1961).

Moreover, the larger molecules are susceptible to non-random degradation to smaller molecules of at least three different size ranges. The integral sedimentation analyses and the molecular weight determinations suggest quite a high degree of homogeneity among the molecules in these three groups. Accordingly we may consider each as a new molecular species derived from the original pair of molecules.

The 8.8 and 4.4S molecules for which the molecular weights have been measured obey the empirical expression $s^0 = kM^{0.5}$ relating sedimentation coefficient and molecular weight. The same relationship holds also for the two original molecules. By implication, therefore, the third components of 13.1S would have a molecular weight close to 300,000. There is thus an interesting quantization of molecular weights among the five components under discussion. Starting with the limiting size 4.4S molecule a fourfold increase in molecular weight gives the 8.8S component. Further increases by factors of two lead to the 13.1S component and the 18S and 28S molecules successively. All the RNA molecules observed during this study fall into one of these five classes.

The degradation of RNA molecules could be ascribed to at least three possible mechanisms; contaminating nucleases, breaks at weak points of the molecule, or a depolymerization into subunits. The first alternative would seem to be a rather unlikely one. Contaminating nuclease action would not be expected to lead to discrete components, quantized in terms of molecular weight or to an ultimate unit, although it is possible that the secondary structure is such as to expose regularly spaced covalent linkages susceptible to nuclease action. When bacterial RNAase

is intentionally added it does not produce a polynucleotide chain of about 100 units but the reaction goes to completion resulting largely in mononucleotides.

Alternatively the folding of the molecule could result in bonds, again regularly spaced, that are more strained than most of the phosphodiester linkages and thus more susceptible to heating, alteration in the ionic environment, or extent of hydrogen bonding. Such linkages may be of a different type from those commonly supposed to be universal in polynucleotide chains. On the basis of the present results it is not possible to distinguish between such a non-random degradation and the existence of real subunits. One possible way of making this distinction is to examine the kinetics of ribosomal RNA synthesis *in vivo*. These experiments are discussed in the subsequent paper (McCarthy and Aronson, 1961).

One of the authors (B. J. McCarthy) would like to express his gratitude to Professor Paul Doty of Harvard University, in whose laboratory the molecular weight determinations were made, for his helpful advice and discussion.

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Comment. The Harvard group has presented evidence showing that the degradation of ribosomal RNA is indeed the result of nuclease activity (H. Boedtker, W. Moller, and E. Klemperer, Nature, 194, 444, 1962). The evidence presented here that the chain breakage occurs at particular points along the molecule is consistent with recent studies of the structure of ribosomal RNA. Langridge (R. Langridge, Science, 140, 1000, 1963) has described a regular pattern in ribosomal structure arising from the alternation of double helical and flexible regions in the RNA molecule. Timasheff et al. (S. N. Timasheff, J. Wits, and V. Luzzatti, Biophys. J., 1, 525, 1961) arrive at similar conclusions from X-ray studies of isolated ribosomal RNA. Both suggest double helical regions of RNA of a length between 50 and 150 Å. On this model traces of nuclease might be expected to show strong preference for the nonhelical regions so that the degradation products would be of more or less regular sizes. Particularly where the nuclease attack occurs before extraction of the RNA from the ribosome the exposed sections of the RNA molecule susceptible to the enzyme might be a very restricted part of the total. Brian J. McCarthy.

III.C.9 The Kinetics of the Synthesis of Ribosomal RNA in *E. coli*

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ABSTRACT The kinetics of the synthesis of ribosomal RNA in *E. coli* has been studied using C^{14} -uracil as tracer. Two fractions of RNA having sedimentation constants between 4 and 8S have kinetic behavior consistent with roles of precursors. The first consists of a very small proportion of the RNA found in the 100,000 g supernatant after ribosomes have been removed. It has been separated from the soluble RNA present in much larger quantities by chromatography on DEAE-cellulose columns. The size and magnitude of flow through this fraction are consistent with it being precursor to a large part of the ribosomal RNA.

A fraction of ribosomal RNA of similar size is also found in the ribosomes. This fraction is 5 to 10 per cent of the total ribosomal RNA and a much higher proportion of the RNA of the 20S and 30S ribosomes present in the cell extract. The rate of incorporation of label into this fraction and into the main fractions of ribosomal RNA of 18S and 28S suggests that the small molecules are the precursors of the large molecules. Measurements of the rate of labeling of the 20, 30, and 50S ribosomes made at corresponding times indicate that ribosome synthesis occurs by concurrent conversion of small to large molecules of RNA and small to large ribosomes.

INTRODUCTION

It has been demonstrated (Aronson and McCarthy, 1961) that although most ribosomal RNA occurs as large molecules having molecular weights of approximately 1.2×10^6 and 5.5×10^5 , there are also present in ribosomes small quantities of RNA of lower molecular weight. In addition it has proved possible to degrade all the RNA by gentle procedures to discrete molecules of about 30,000 molecular weight. Together these observations could suggest an important role of small molecules of RNA as precursors to the large molecules. In this paper the presence of the small RNA molecules in the ribosomes of various sizes is studied together with their kinetic behavior. The small molecules of RNA are shown to be more rapidly labeled than the bulk of large molecules.

MATERIALS AND METHODS

The methods of growing bacteria have already been detailed (Aronson and McCarthy, 1961). The strains of *E. coli* used were ML 30 and 15 T⁻A⁻U⁻ which requires thymine, arginine, and uracil for growth. The latter strain was always grown in the presence of thymine and arginine. The C¹⁴-uracil used to observe RNA synthesis was obtained from the California Corporation for Biochemical Research and had specific activities in the range 2 to 4 mc/mm. Cells uniformly labeled with P³² were prepared by growing overnight in a glucose-salts medium, buffered with tris, containing 1 gm/liter nutrient broth to supply carrier phosphate and 1 to 2 mc P³² as orthophosphate.

Ribosomes and RNA were fractionated according to size by means of sucrose gradient centrifugation (Britten and Roberts, 1960).

Radioactivity present in macromolecules was estimated after adding trichloroacetic acid (TCA) (to 5 per cent) to the sample and passing through a millipore filter. This technique also served to follow rates of incorporation of tracers (Britten, Roberts, and French, 1955; McQuillen, Roberts, and Britten, 1959).

RESULTS

1. Kinetics of a Free Ribosomal RNA Fraction

(a) *Separation of Ribosomal RNA and S-RNA.* If ribosomal particles are built up by sequential additions of protein and RNA so that the smaller particles are precursors to the larger, it might be possible to detect ribosomal RNA before it becomes a part of a nucleoprotein. The proof of its existence would depend on an adequate method of differentiating it from the soluble RNA (S-RNA) which is present in much larger quantities. Sedimentation analysis would not be suitable if such a precursor was of a low molecular weight close to that of S-RNA. There-

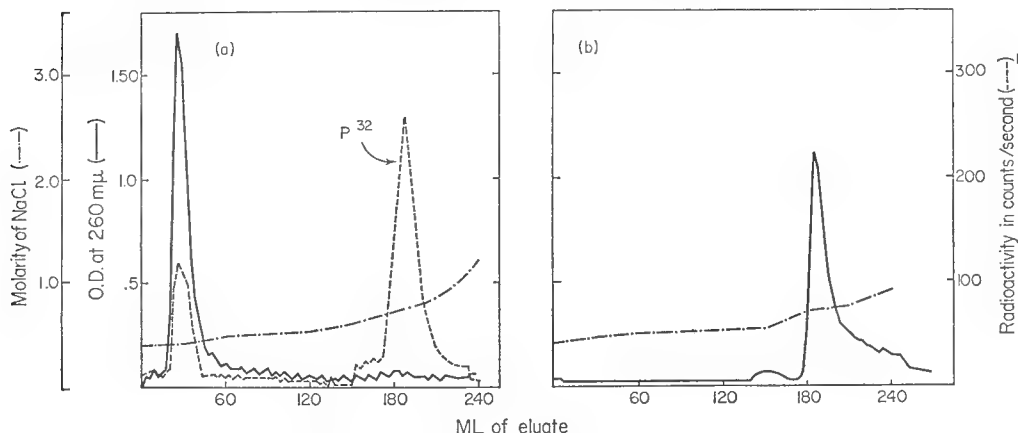


FIGURE 1 Chromatography on a DEAE-cellulose column. Non-linear sodium chloride gradient from 0.4 to 2.0 M in buffer (tris 0.01 M, succinic acid 0.004 M, magnesium acetate 0.01 M, pH 7.4). (a) A mixture of unlabeled S-RNA and P³² total RNA, phenol-extracted from magnesium-starved cells. (b) 28S and 18S RNA, phenol-extracted from 70S particles.

fore a chromatographic method of separation was tried to differentiate ribosomal RNA from S-RNA. It proved possible to obtain complete resolution between S-RNA and ribosomal RNA by chromatography on a DEAE-cellulose column. Fig. 1 *a* shows the result of elution of a mixture of a P^{32} -labeled total of RNA fraction and a large excess of unlabeled soluble RNA. The labeled cells had been exhaustively starved of magnesium. This procedure degrades the ribosomes to their constituent protein and RNA moieties (McCarthy, 1959) and the RNA to molecules of 4 to 5S (Aronson and McCarthy, 1961). The labeled RNA therefore consisted of a mixture of ribosomal RNA and S-RNA of closely similar sedimentation constants. Phenol-extracted unlabeled S-RNA was added in excess as carrier. The eluting buffer contained tris 0.01 M, succinic acid 0.004 M, magnesium acetate 0.01 M, pH 7.4 with a non-linear gradient of sodium chloride from 0.4 to 2.0 M. The salt gradient was made with the aid of a seven chamber device of the type used by Peterson and Sober (1959). Virtually all the ultraviolet absorbing material was eluted at the front together with the small fraction of P^{32} corresponding to S-RNA originally in the labeled cells. Most of the radioactivity associated with ribosomal RNA was eluted near 0.8 M.

The salt concentration at which ribosomal RNA is eluted does not seem to

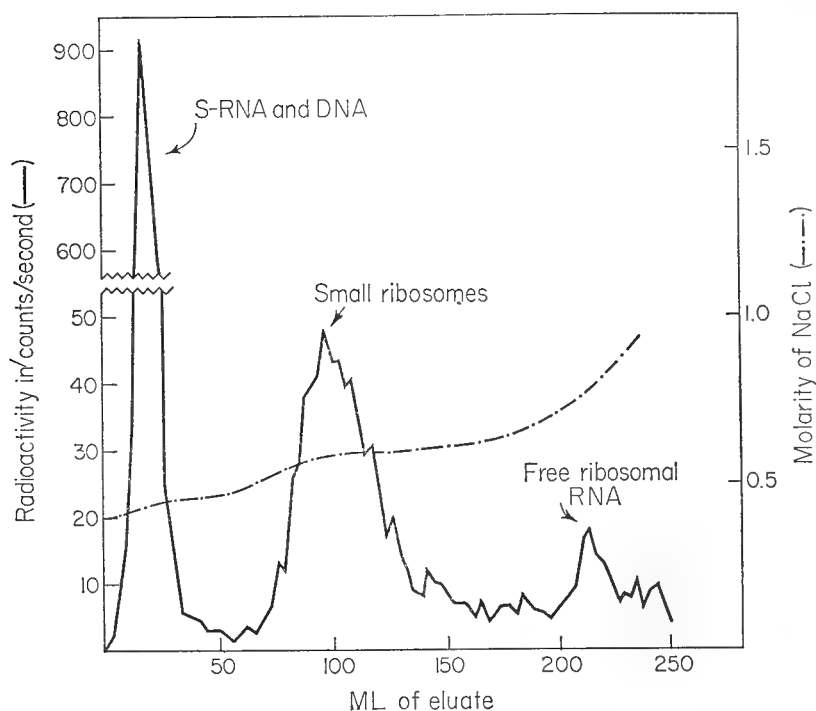


FIGURE 2 Chromatography on a DEAE-cellulose column. Non-linear sodium chloride gradient from 0.4 to 2.0 M in buffer (tris 0.01 M, succinic acid 0.004 M, magnesium acetate 0.01 M, pH 7.4). 40K 90 minutes SN of P^{32} randomly labeled cell juice.

depend very strongly on its molecular weight. Fig. 1b shows the elution pattern for a mixture of 28S and 18S RNA prepared by the phenol method from 70S particles. Again the RNA is eluted in the region of 0.8 M salt.

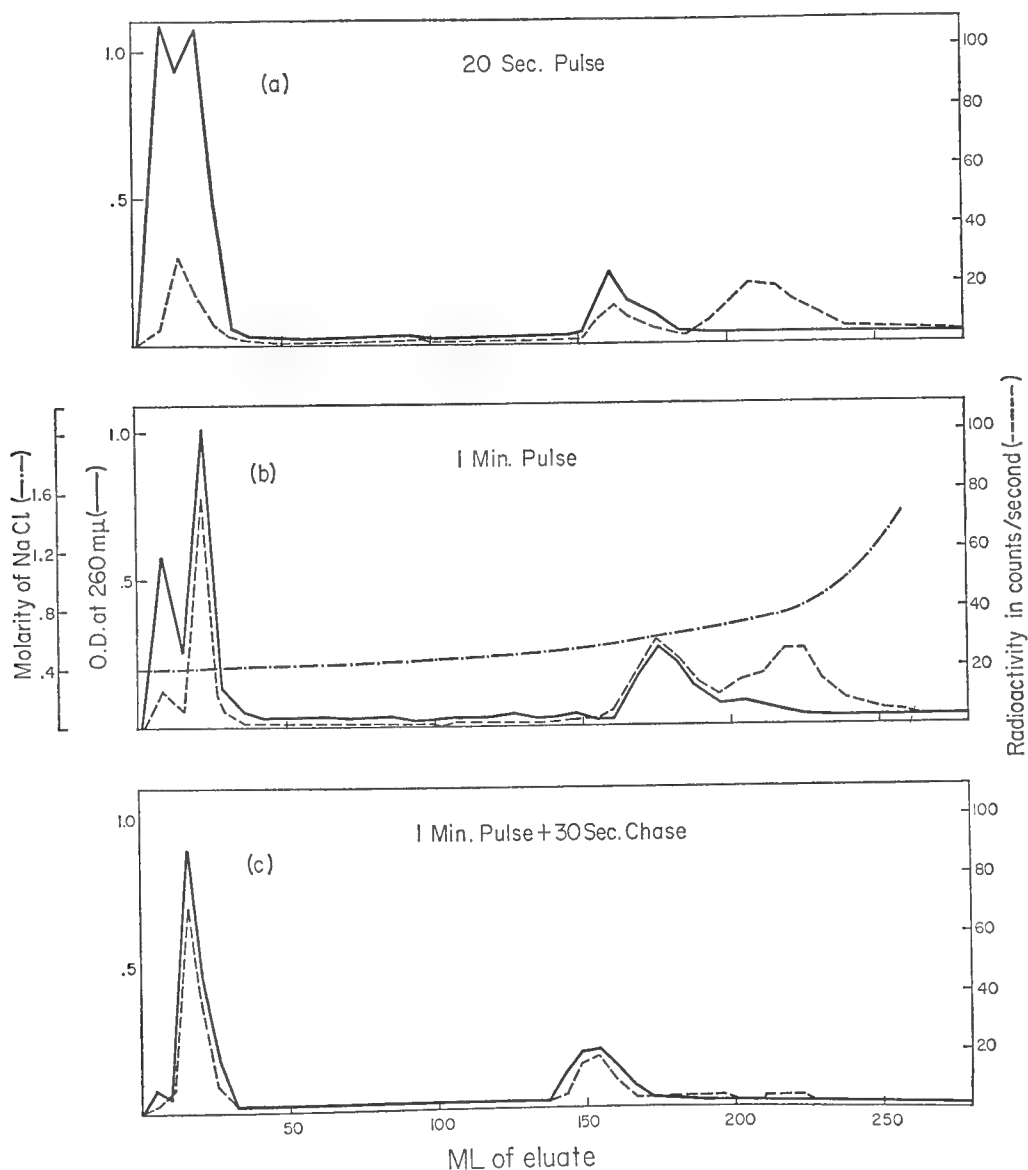


FIGURE 3 Chromatography on a DEAE-cellulose column of a 37K 180 minute SWB SN of three cell juices. Non-linear sodium chloride gradient from 0.4 to 2.0 M in buffer (tris 0.01 M, succinic acid 0.004 M, magnesium acetate 0.01 M, pH 7.4). (a) Cells given a 20 second exposure to C^{14} -uracil (0.5 μ g/ml). (b) Cells given a 1 minute exposure to C^{14} -uracil. (c) Cells given a 1 minute exposure to C^{14} -uracil (0.5 μ g/ml) followed by a 30 second chase in C^{12} -uridine and cytidine in excess (100 μ g/ml of each).

Since soluble and ribosomal RNA's can be readily separated by this technique, it is possible to chromatograph cell extracts to determine whether any free ribosomal RNA is present. An extract from cells uniformly labeled with P^{32} was first centrifuged $40K^1$ for 180 minutes ($40K\ 180$) to remove the bulk of the ribosomes. A sample of the supernatant was then chromatographed on a DEAE-cellulose column employing the same non-linear salt gradient (Fig. 2). The first peak contains a mixture of S-RNA and DNA, the second elutes at a salt concentration characteristic of small ribosomes and represents the few left in the supernatant after centrifugation, and the third small peak elutes in the region of free ribosomal RNA. Treatment of the first peak with DNAase showed 40 per cent of the P^{32} to be in DNA, so that the remaining 60 per cent would be in S-RNA.

As the sedimentation constants of S-RNA and the free ribosomal RNA are similar, as will be shown later, the same proportion of each should remain in solution after centrifugation. It is therefore justifiable to estimate the quantity of ribosomal RNA by comparison with the known quantity of S-RNA. Summing the radioactivity under the first and third peaks shows that the ribosomal RNA is approximately 1.5 per cent of the S-RNA. Accordingly, the quantity of free ribosomal RNA would be about 0.4 mg/gm dry weight of cells. If this were precursor material to all the particle-bound RNA, it would provide material for about 15 seconds' growth for cells growing with a generation time of about 1 hour. Consequently, studies with labeled uracil should reveal whether this material has the kinetic characteristics of a precursor or whether this small quantity of free ribosomal RNA arises from degradation of particles.

(b) *Pulse Studies with C^{14} -Uracil.* The uracil-requiring mutant (15 T-A-U-) was allowed to incorporate C^{14} -uracil for periods of 20 seconds or 1 minute. The majority of the ribosomes were removed from the two cell juices obtained by means of a $37K\ 180$ minute spin in the swinging bucket rotor. The resulting supernatants were analyzed on DEAE-cellulose columns (Figs. 3 a, 3 b). Radioactive uracil appears in three distinct regions. The first and third have been identified as S-RNA and ribosomal RNA, respectively, by the salt concentrations at the peaks. The second component is due to residual small ribosomes.

Sedimentation analysis of the same 1 minute pulse supernatant showed that most of the radioactivity appeared in a single peak corresponding to about 4 to 8S (Fig. 4). The 25 per cent of the radioactivity found in the pellet at the bottom of the tube can be attributed to the residual small particles in the preparation which must have sedimented since they have sedimentation constants of at least 20S. The other 75 per cent of the radioactivity must include both S-RNA and ribosomal RNA so that both must be in the range of 4 to 8S.

In Figs. 3 a and 3 b, it can be seen that the total label in the first peak increased with time, showing continued synthesis of S-RNA. The specific radioactivity of

¹ K, 1000 R.P.M.

the middle component, representing a mixture of residual small ribosomes, rises only slightly when compared to the S-RNA. The third component, the presumed precursor, has a constant quantity of label after 20 seconds. Such data are consistent with the small precursor pool. If it can be shown that this radioactivity is lost from the pool as quickly as it enters (20 seconds) then this fraction can be assumed to be either an intermediate in a kinetic sequence or rapidly degraded back to nucleotides. To determine whether label is rapidly lost from this fraction, extracts from cells exposed to radioactive uracil for a very short time followed by dilution with a large excess of unlabeled uridine and cytidine were analyzed.

Unfortunately, the mononucleotide pool which cells rapidly accumulate after

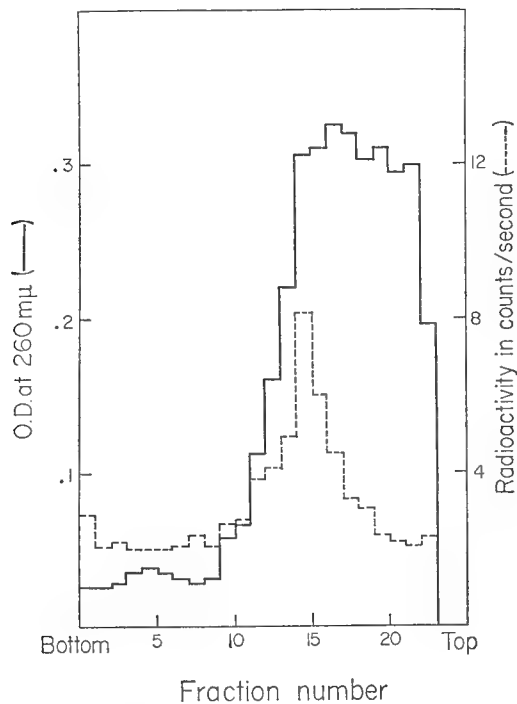


FIGURE 4 Sedimentation analysis of a 37K 180 minute SN of an extract from cells given a 1 minute pulse label with C^{14} -uracil. Most of the ultraviolet-absorbing material between fractions 17 and 24 is protein. Centrifugation 30K 16 hours. An object of about 6S would be expected to peak in the middle of the tube.

a very brief exposure to exogenous C^{14} -uracil is sufficient for some 5 minutes' growth. The addition of a gross excess of non-radioactive uracil and cytosine fails to dilute out the pool. Thus, under normal conditions of growth, a 5 to 10 minute dilution period ("chase") of C^{14} -uracil is the minimum possible. It is, however, possible to surmount this difficulty and to obtain rapid cessation of further incorporation of label into polynucleotide. Bacterial cells will grow in a medium of high osmotic strength without any appreciable loss of metabolic ability or diminution of

growth rate. If such a culture is quickly added to an equal volume of distilled water, the resulting osmotic shock is sufficient to cause the cells to lose their pools of amino acids and nucleotides almost completely. This technique makes possible the rapid cessation of further incorporation of label.

Uracil-requiring cells were grown in C^{14} -uracil ($0.5 \mu\text{g/ml}$) for 1 minute in C medium containing 0.25 M sucrose, and then quickly poured into an equal volume of distilled water. After a few seconds, a two hundred-fold excess of unlabeled uridine and cytidine was added and quickly followed by the salts necessary to bring the medium back to the original composition. Prior experiments had shown that such manipulations did not cause a delay in the resumption of protein or RNA synthesis. Measurement of the uptake of C^{14} -uracil showed a rapid decrease in the

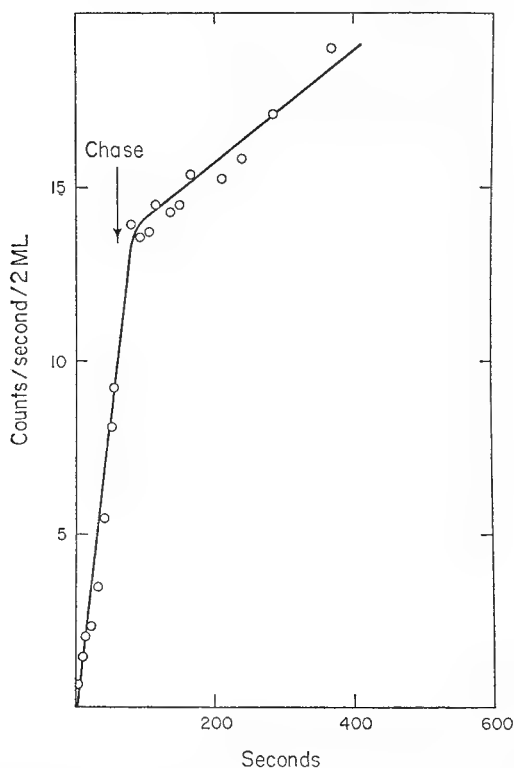


FIGURE 5 The incorporation of C^{14} -uracil ($0.5 \mu\text{g/ml}$) by *E. coli* T⁻A⁻U⁻ into a TCA-precipitable fraction. Cells growing at 37° C in a maltose-salts medium containing 0.25 M sucrose. At 1 minute the cells were diluted by pouring into an equal volume of distilled water. This was followed by an addition of 100 $\mu\text{g/ml}$ each of C^{12} -uridine and cytidine and the salts necessary to bring the medium back to the normal composition.

rate of incorporation by a factor of 12 (Fig. 5). Samples were harvested after chasing for various times, chilled, and processed in the usual manner.

Fractionation of the juice from cells given a 30 second chase by this technique

after a 1 minute uracil pulse, showed very little label left in the ribosomal RNA region of the elution diagram (Fig. 3c). Thus, this pool does have the properties of an intermediate, being rapidly labeled and just as rapidly emptied. Although neither the estimate of the steady state pool size nor the turnover time are accurate to better than a factor of two, the existence of such a pool does suggest that at least a large percentage of the particle-bound RNA could pass through a pool of free ribosomal RNA before it is assembled into nucleoprotein.

2. Newly Synthesized RNA in Ribosomes

The state of the newly synthesized RNA actually within nucleoprotein particles was then investigated. Total particle fractions were prepared by centrifuging a cell extract in the swinging bucket rotor at 37K for 3 hours. At least 90 per cent of the particle RNA was collected by this means. The pellets were then extracted with phenol to obtain the RNA and fractionated into the various sized components on the sucrose gradient. Fig. 6 compares the distribution of radioactivity in the ribo-

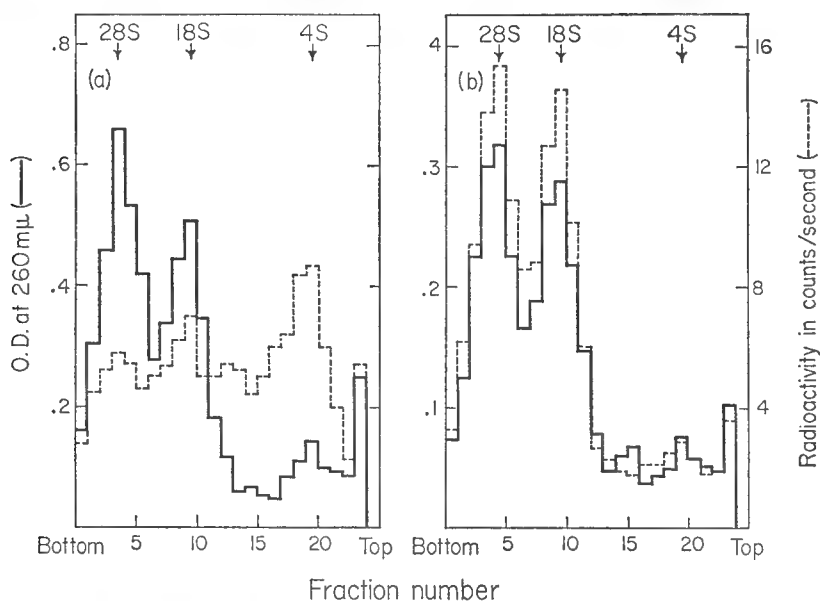


FIGURE 6 Sedimentation analysis of RNA extracted from ribosomes. (a) From cells given a 30 second exposure to C^{14} -uracil (0.5 μ g/ml). (b) From cells given a 30 second exposure to C^{14} -uracil (0.5 μ g/ml) followed by 20 minutes' growth in C^{12} -uracil (100 μ g/ml). Centrifugation 37K 280 minutes.

somal RNA from cells given a 30 second C^{14} -uracil pulse with that of the same cells given a subsequent 20 minute chase with C^{12} -uracil. It is immediately apparent that low molecular weight RNA of between 4 and 8S is most highly labeled at early times and that at the subsequent time point virtually all of the radioactivity is found

in the 18 and 28S fractions. There can be little contamination of the particles by soluble RNA, as much of its radioactivity would persist and be present in the 4S region after the 20 minute chase. Therefore, it may be concluded that small molecules of RNA enter nucleoprotein in that form and that there seems to be a second process by which labeled material of this component passes on into the larger molecules.

Since small RNA components are prominent in short term labeling experiments, the distribution of the small RNA components among the various sized ribosomes was determined. Each of the four ribosome classes (70S, 50S, 30S, 20S) was isolated from two extracts, one from cells which had been exposed to C^{14} -uracil for 1 minute and the other from uniformly labeled cells. A 40K 180 minute pellet was fractionated by sedimentation to separate the 70, 50, 30, and 20S ribosomes (Fig. 7). Characterization of the RNA from each of these fractions was then made

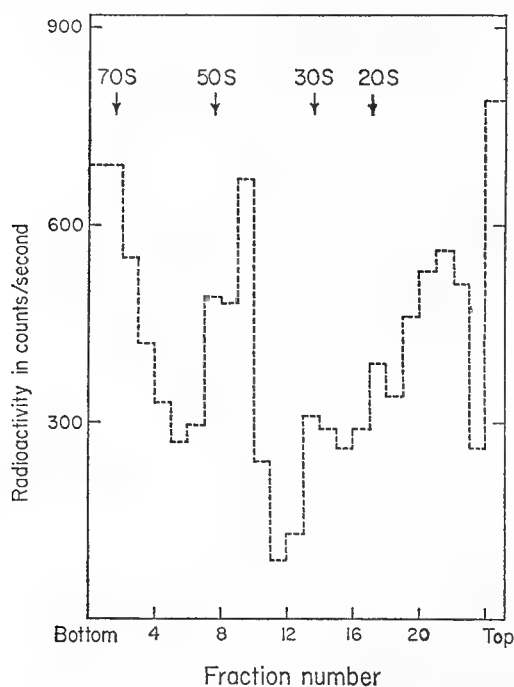


FIGURE 7 Sedimentation analysis of C^{14} -uracil uniformly labeled ribosomes (40K 180 P). The peaks correspond to 70S, 50S, 30S, and 20S ribosomes. The fractions from which RNA was extracted were 70S 2-5, 50S 8-10, 30S 14 and 15 and 20S 17 and 18. Centrifugation 37K 120 minutes.

by means of a second sucrose gradient separation (Fig. 8). This analysis of the uniformly labeled RNA's of the different particles is analogous to the schlieren studies already described (Aronson and McCarthy, 1961) but it is sufficiently more sensitive for it to permit the study of the RNA of the less abundant "native"

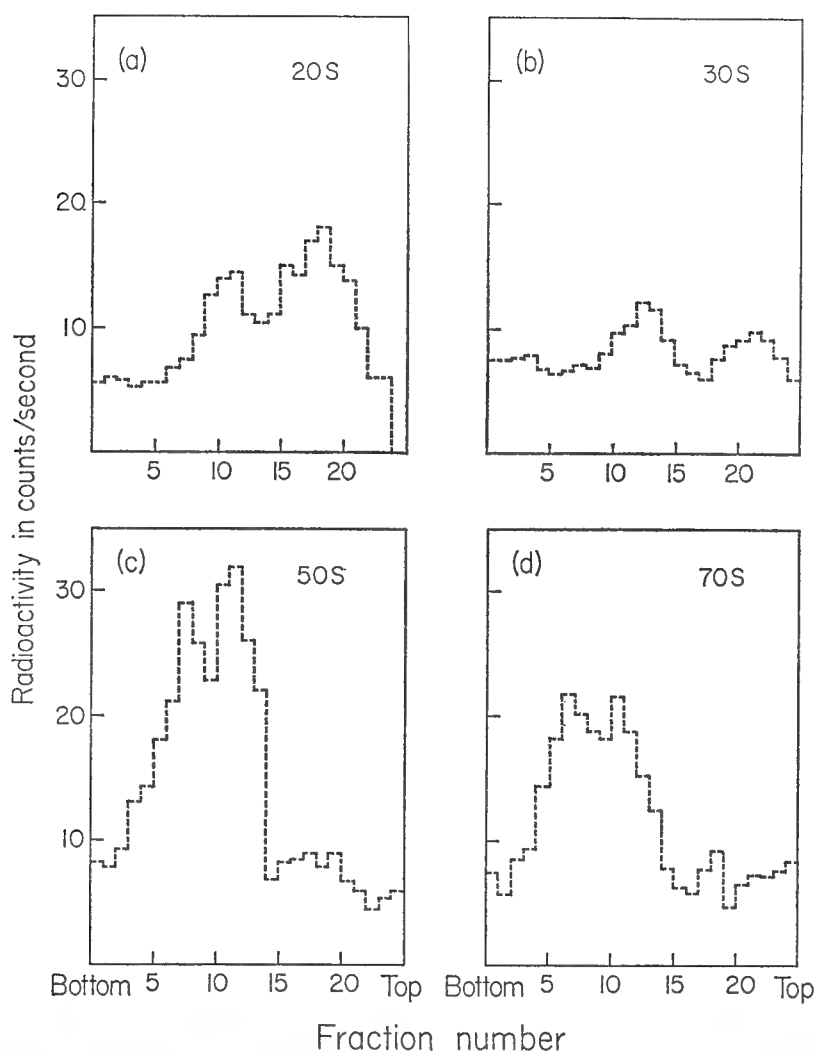


FIGURE 8 Sedimentation analysis of RNA extracted from C^{14} -uracil randomly labeled ribosomes. (a) 20S. (b) 30S. (c) 50S. (d) 70S. Ribosome fractions obtained by the separation shown in Fig. 7. Centrifugation 37K 240 minutes. An object of 18S would be expected to peak in the middle of the tube.

50, 30, and 20S particles. The native 50S particles are made up mostly of 28 and 18S RNA, but they also contain some RNA of 4 to 8S. Fig. 8 *a* showing the RNA extracted from 20S particles, demonstrates RNA of both 18S and 4 to 8S. As previously discussed (Aronson and McCarthy, 1961), the larger RNA component cannot be part of a single 20S particle. The fact that the 20S particles contain the highest proportion of low molecular weight RNA would be in close accord with a role as precursor of some, at least, of the larger particles.

The analogous experiments with 1 minute pulse-labeled ribosomes showed the distribution of newly formed RNA. Even in the 70S particles about one-third of the

radioactivity appeared as material of low sedimentation constant (4 to 8S) (Fig. 9). The 50S particles contained only traces of radioactive 18 and 28S RNA, the radioactivity appearing mainly in 4S and 12S RNA. The labeled RNA of both the 20 and 30S particles was mainly 4 to 8S. It is evident that the 4 to 8S material is not confined to any one class of ribosome.

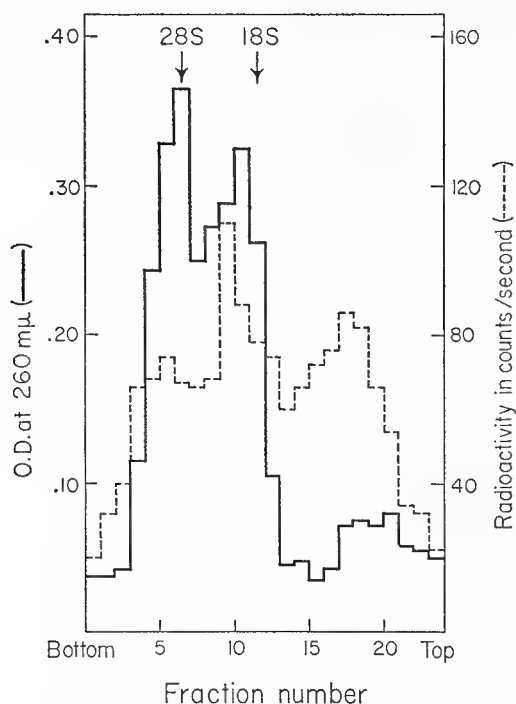


FIGURE 9 Sedimentation analysis of the RNA extracted from purified 70S ribosomes of cells given a 1 minute exposure to C^{14} -uracil. Centrifugation 37K 240 minutes.

To examine in more detail the fate of this low molecular weight fraction of the ribosomal RNA, a series of different length uracil pulses and chases were studied. Fig. 10 shows the analysis of RNA derived from particles of cells given 20, 40, and 60 second pulses of C^{14} -uracil and a 60 second pulse followed by 5 minute and 10 minute chases. The ribosomes were prepared by a 40K 180 minute centrifugation so that the yield of the smaller ribosomes was not complete. The small 4 to 8S RNA becomes labeled very quickly, and the specific radioactivity continues to increase linearly for at least 1 minute.

In Fig. 11 the total label in the three main classes of RNA 28S, 18S, and 4 to 8S is plotted as a function of time. The label in the smallest components has not reached a saturation value by 1 minute and, therefore, the pool of such material must be greater than a minute's supply if it is an intermediate. On the other hand, there is evidence of a lag in the flow of label into the 18S and 28S regions suggesting the existence of a precursor. On chasing the label is lost from the small com-

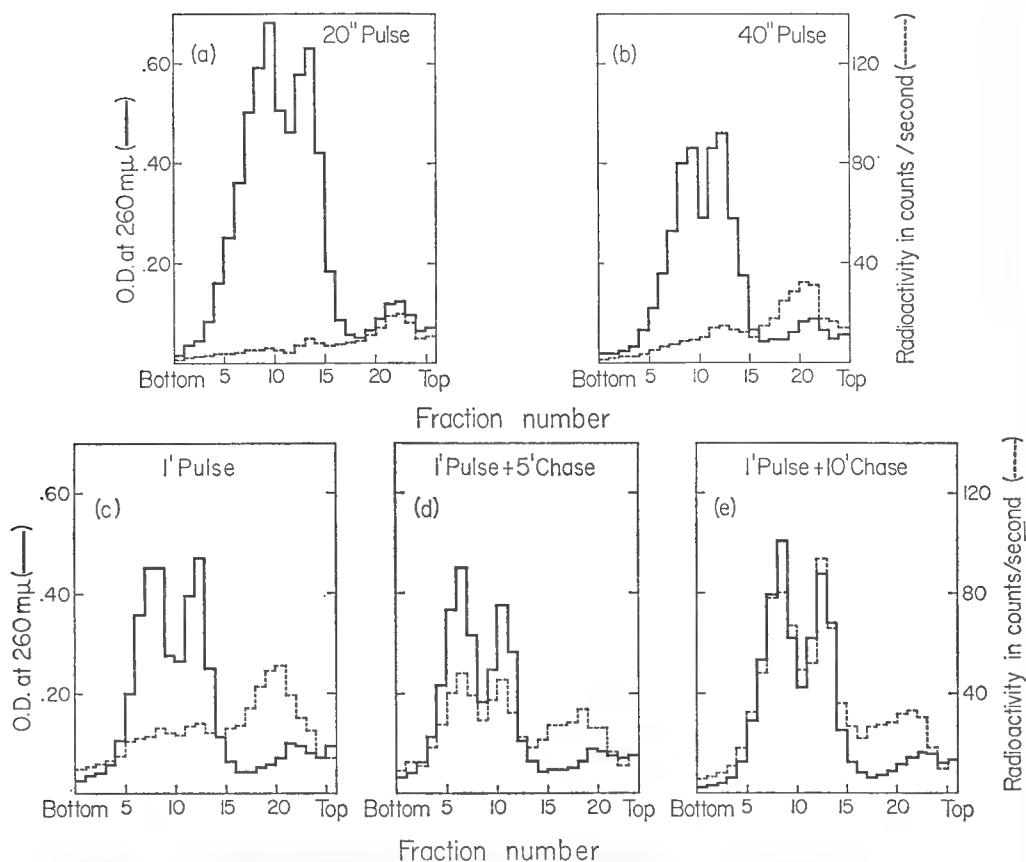


FIGURE 10 Sedimentation analysis of the RNA extracted from ribosomes. (a) From cells given a 20 second exposure to C^{14} -uracil (0.5 $\mu\text{g}/\text{ml}$). (b) 40 second exposure. (c) 1 minute exposure. (d) 1 minute exposure to C^{14} -uracil (0.5 $\mu\text{g}/\text{ml}$) followed by a 5 minute period in C^{13} -uridine and cytidine in excess (100 $\mu\text{g}/\text{ml}$ of each). (e) 1 minute exposure to C^{14} -uracil followed by a 10 minute period in C^{13} -uridine and cytidine. Centrifugation 37K 240 minutes. An object of 18S would be expected to peak in the middle of the tube.

ponents to reach a constant value by about 5 minutes. At the same time, more label flows into the large components.

The distribution of ultraviolet-absorbing material in Figs. 6 and 10 shows that about 5 per cent of the ribosomal RNA is in the form of 4 to 8S material. With a generation time of 50 to 60 minutes the RNA increases at about 2 per cent per 100 seconds. Thus if the 4 to 8S RNA were entirely precursor to the 18S and 28S components, its quantity would be sufficient to provide material for about 4 minutes' growth. Accordingly it is possible to consider how well the observed data of Fig. 11 fit with such a precursor-product relationship, as opposed to complete independence of the synthesis of 4 to 8S RNA and the larger 18S and 28S.

The fact that the label in the 4 to 8S fraction decreases by only a factor of three

on chasing could be a result of the inefficient chase. A 4 minute pool of 4 to 8S RNA would have reached only one-quarter of its eventual specific radioactivity after a 1 minute labeling. But the chase itself decreased the rate of uptake of label by only a factor of twelve. Therefore the chase would reduce the specific radioactivity or the total label in the 4 to 8S pool by a factor of three from one-quarter to one-twelfth.

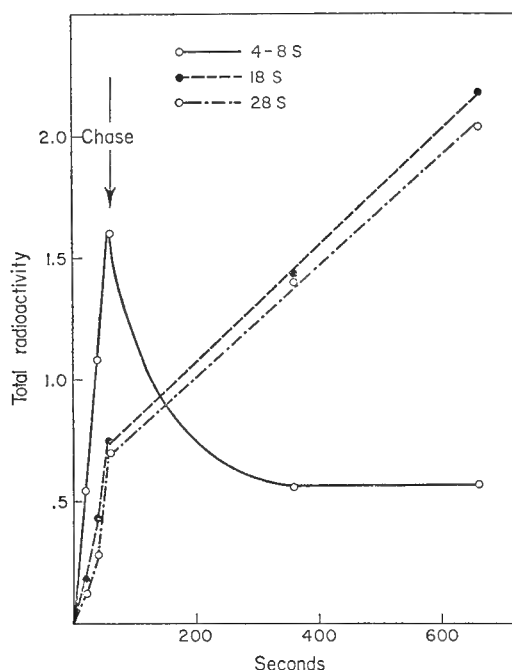


FIGURE 11 The total radioactivity in the three main fractions of ribosomal RNA of cells given a 1 minute exposure to C^{14} -uracil followed by a 10 minute period in C^{12} -uridine and cytidine. Data from Fig. 10.

3. Kinetics of Ribosome Synthesis

To correlate these observations on the synthesis of RNA with a scheme of particle synthesis, additional kinetic studies were made of the incorporation of C^{14} -uracil into the ribosomes. Such experiments represented a continuation of a series of pulse labeling and chasing studies performed in this laboratory over the past 3 years (Roberts *et al.*, 1958, 1959, 1960). For the most part, either S^{35} or P^{32} had been previously employed as a tracer. C^{14} -uracil was used in this series to avoid the kinetic delays introduced by the large pool of low molecular weight phosphorus compounds. Some effort was made to obtain results using pulses and chases of the same times as had been employed in the RNA studies so that a correlation could be made between particle kinetics and RNA kinetics. In many

cases, part of the sample was fractionated in the ribosome form and another as free RNA.

Sedimentation analyses of a total ribosome pellet (37K 180 P) in the region of the 50S, 30S, and 20S ribosomes do not show well resolved peaks corresponding to these objects. The interpretation of such analyses in terms of the labeling of these specific objects therefore requires some justification. This difficulty arises because of the presence of a large quantity of 70S ribosomes at the bottom of the tube. It is possible to remove most of these by suitable centrifugation and then to concentrate the remaining 50S, 30S, and 20S ribosomes by a further centrifugation. Subsequent sedimentation analyses then show much clearer indications of three peaks. In the present case, however, it seemed that the more prolonged manipulation of the preparation and the consequent increased possibility of degradation would more than nullify the advantages of a cleaner separation.

On the other hand, there is no doubt that the small ribosomes are present and separated by this technique. The relative proportions of the 50S, 30S, and 20S ribosomes indicated by the ultraviolet absorption agree with those determined in the model E analytical ultracentrifuge. These ribosomes represent about 10, 5, and 5 per cent respectively of the total ribosomal material of the cell juice. Fractions taken from the peaks of the swinging bucket analyses or from where the ribosome peak is expected and analyzed in the model E analytical ultracentrifuge show not only the presence of the particular ribosome expected for that fraction, but also that these ribosomes account for most of the ultraviolet absorption. In view of this it seems justifiable to interpret the results of these sedimentation analyses in terms of the three classes of ribosomes, rather than in terms of a heterogeneous mixture.

The results to be presented have been analyzed mainly on the basis of the distribution of radioactivity. Fig. 12 represents sedimentation analyses of five C^{14} -uracil pulse-labeled total ribosome pellets collected by a 37K 180 minute centrifugation. The five samples were taken after a 20 second, a 40 second, and a 1 minute pulse, and a 1 minute pulse followed by 1 and 2 minute chases. Specific radioactivities were calculated to normalize the data for differences in the quantity of material analyzed, and to summarize the results (Fig. 13).

These early pulse-labeling studies fail to elucidate the exact relationship among the 20, 30, and 50S ribosomes for there are no striking differences among the rates of labeling of the three regions corresponding to the three ribosome groups. At the earliest time, 20 seconds, the radioactivity is more or less equally distributed among the three regions. Although the specific radioactivities of the 20S and 30S seem to be very close throughout the three pulse points, that of the 50S lags behind during the 1st minute. The difference in specific radioactivity obtained from the data of Fig. 12 is consistent with the equal distribution of total radioactivity among the three regions, and with the greater quantity of 50S ribosomes present in a cell extract.

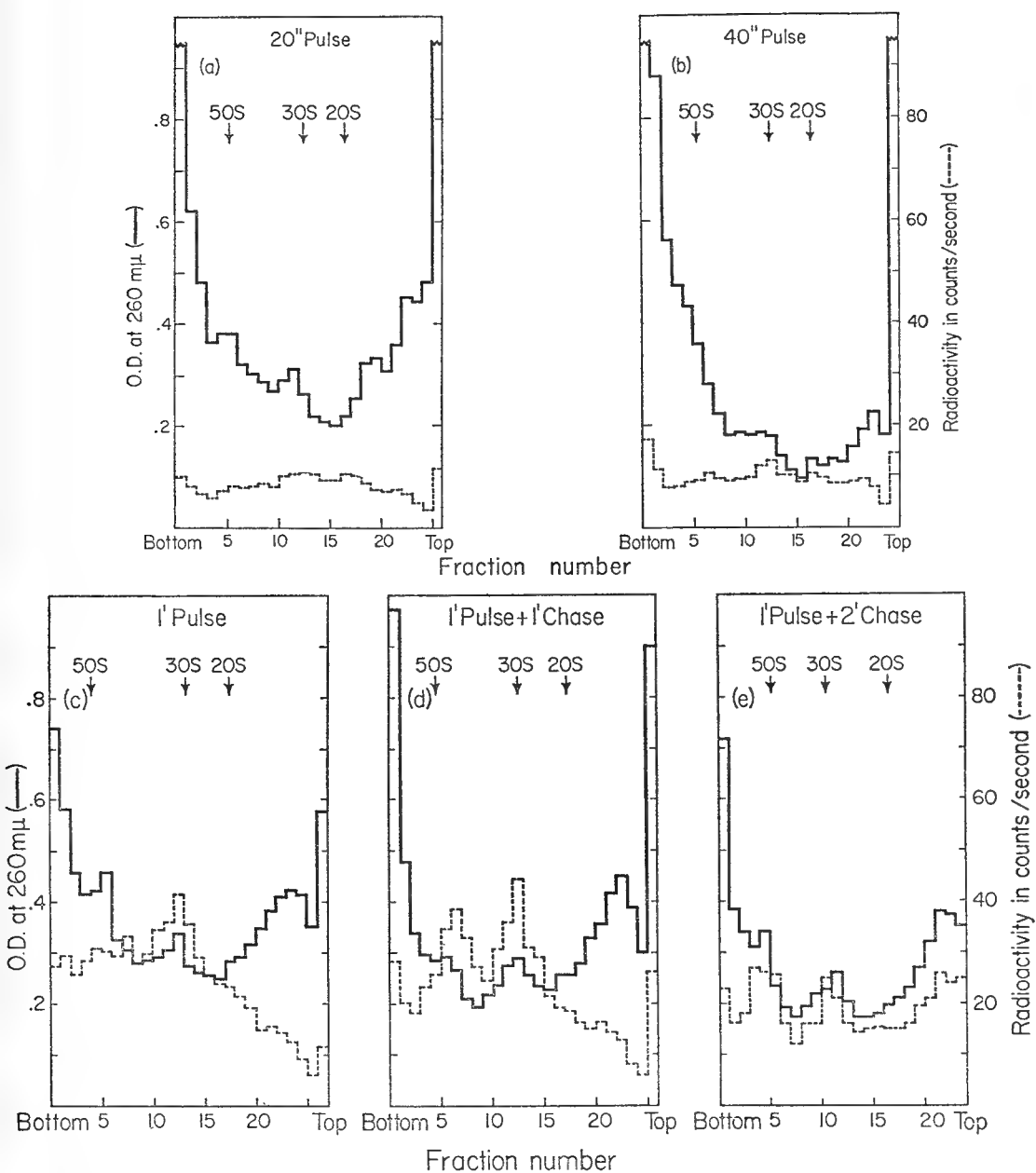


FIGURE 12 Sedimentation analysis of five total ribosome pellets (37K 180 P). (a) From cells given a 20 second exposure to C^{14} -uracil (0.5 μ g/ml). (b) 40 second exposure. (c) 1 minute exposure. (d) 1 minute exposure to C^{14} -uracil (0.5 μ g/ml) followed by a 1 minute period in excess C^{12} -uridine and cytidine (100 μ g/ml of each). (e) 1 minute exposure to C^{14} -uracil followed by a 2 minute period in C^{12} -uridine and cytidine. Centrifugation 37K 150 minutes.

The distribution of label among the three ribosomes at the earliest times indicates three separate points of entry of newly synthesized RNA. An independent synthesis of each of the three small ribosomes would require a distribution of early radioactivity in proportion to the quantity of the three ribosomes. The radioactivity associated with the 50S at early times would then be twice that observed. It seems more reasonable, therefore, to suggest that the ribosomes are built by addition of RNA and protein to a preexistent core so that the 20S becomes a 30S and 30S becomes a 50S. This has already been proposed by Roberts *et al.* (1959) on the basis of early labeling studies using P^{32} and S^{35} as tracer, the results of which bear much similarity to those discussed here.

Of further interest is the proportional and rapid loss of specific radioactivity of the 50 and 30S components during the 2nd minute of chasing (Figs. 12 and 13). If the synthesis of 70S particles from 30S and 50S were a one way process, then the pools of these smaller particles based on the steady state quantities relative to 70S should empty only about five or six times per generation. The higher rate

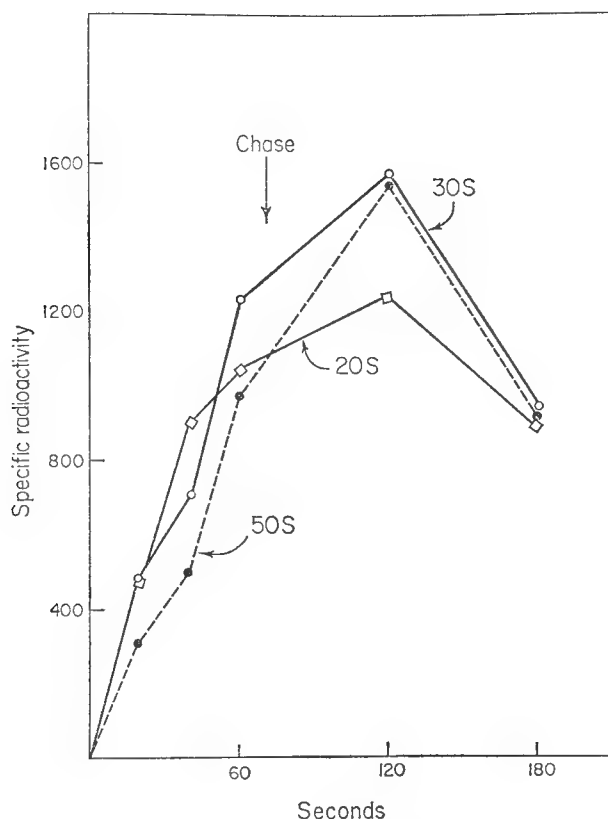


FIGURE 13 The specific radioactivities of the 20S, 30S, and 50S ribosomes of cells given a 1 minute exposure to C^{14} -uracil followed by a 2 minute period in excess C^{12} -uridine and cytidine. Data from Fig. 12.

of loss of specific radioactivity observed suggests that the new particles are diluted by an equal number from the 70S fraction about every $1\frac{1}{2}$ to 2 minutes so that only about one-fifth of the 30S and 50S pools are really precursors. Since roughly 80 per cent of the ribosomal material is in the form of 70S and 100S particles, we can make an estimate of about 4 to 6 minutes as the average lifetime of a large particle before it breaks down to its component parts. These calculations are approximate and further experiments are needed to clarify this process.

DISCUSSION

The present studies on the kinetics of RNA synthesis have clearly shown that there are present minor components of between 4 and 8S which are very rapidly labeled. Such material can be demonstrated as free RNA in the 100,000 g supernatant and also as part of the ribonucleoprotein fraction. The former fraction has kinetic characteristics (magnitude of flow and time constant) which are consistent with its being a precursor to the main bulk of ribosomal RNA. The available data are, however, equally consistent with the behavior of an independent RNA fraction unrelated to the synthesis of ribosomal RNA which is rapidly synthesized and subsequently degraded to nucleotides or lost from the cell. At the moment, a choice cannot be made between these two possibilities.

The second component of similar molecular size found in the ribosome fraction is also rapidly labeled although it has a considerably longer turnover time. This extended time is not unreasonable since it is a much more abundant component; *i.e.*, about 5 per cent of the total ribosomal RNA. As with the soluble component it appears that this lower molecular weight RNA is a precursor to the 28S and 18S RNA. However, it is not clear from the crude separation techniques employed how many components are mixed in the 4 to 8S region of the swinging bucket separation. There could be a series of homologous polymers or a mixture of components of each of the two types.

The existence of any appreciable fraction of the pulse uracil label in an RNA fraction which is rapidly exchanging with the nucleotide pool is not supported by the incorporation data of Fig. 5. The sedimentation analysis of ribosomal RNA following a 1 minute uracil pulse shows about one-third of the label in the form of 4 to 8S material. If this were all rapidly turning over by degradation to nucleotide (TCA-soluble) material, the TCA-precipitable counts should fall by 30 per cent very rapidly after the chase was made. This is evidently not the case, so that although label leaves the 4 to 8S material on chasing, it cannot be feeding back into the uracil pool of TCA-soluble compounds. Counts leaving the 4 to 8S component find their way into the 18S and 28S components during the 5 minute chase with non-radioactive uracil. Such a quantitative reutilization of uracil counts would not be expected if there were an intermediate stage during which the label became TCA-soluble again and equilibrated with the uracil pool. In addition, the kinetic delay in

incorporation into 18 and 28S components does suggest the existence of a precursor. On the basis of these two points, then, we are inclined to believe that the 4 to 8S RNA is an intermediate.

The above experiments extend the over-all view of the process of ribosome synthesis about which much information has been collected (Roberts *et al.*, 1958, 1959, 1960). It is already clear that the small quantity of 20S, 30S, and 50S ribosomes present in a cell juice prepared in the presence of 0.01 M magnesium ions is not the result of breakdown of the more abundant 70S ribosomes but is their precursor. Furthermore, as we have seen in these studies there is newly formed RNA in each of these three particle groups at early times. At 20 seconds the uracil label is roughly equally distributed among these three groups. Thus there seem to be three points of entry of new RNA into the ribosome system before the formation of the 70S.

One other general feature of ribosome synthesis is important to the present discussion. Although the small particles are precursors to the large, the process is evidently not unidirectional. A long chase period following a short pulse results in the distribution of the tracers almost equally among all the particle groups (Roberts *et al.*, 1959). Evidently the 70S ribosomes, once formed, are not stable end products but rather break down into their constituent parts again. The short chase periods examined here have shown that the 30S and 50S ribosome pools lose their label very rapidly. This process takes place about ten times as rapidly as is necessary to account for the net synthesis alone. A breakdown process $70S \rightarrow 50S + 30S$ occurring about ten times per generation, with a 70S particle having a lifetime of 5 minutes would fit the observed results.

How may we fit the observations of RNA synthesis with this scheme? It is immediately obvious that if the 20S particle is the ultimate 70S ribosome precursor, ribosomal RNA must exist at early times as molecules of not more than 300,000 molecular weight (the RNA content of a 20S ribosome). In fact, an examination of the RNA of the different ribosome groups has shown that the 20S and 30S particles have a high proportion of low molecular weight RNA. Most of the 5 per cent or so of the postulated ribosomal RNA precursor must therefore be located in these precursor ribosomes. Such a distribution would be expected if the conversion of small to large ribosomes and of small to large RNA molecules are concurrent processes. In addition, the rapid circulation between 30S and 50S, and 70S ribosomes could explain how some of the 4 to 8S pulse-labeled RNA is found in the 70S ribosomes when the pool of such material is about a 5 minutes' supply. A flow of ten times the net increase of material through the 30S and 50S ribosome pools would sweep some low molecular weight RNA on into 70S ribosomes. Therefore, the polymerization of precursor RNA cannot be mandatory for the conversion of 30S and 50S ribosomes to 70S.

In summary, the following scheme for the synthesis of ribosomal RNA may be

presented. Newly polymerized nucleotides appear first in 4 to 8S RNA which can be isolated from the 100,000 *g* supernatant of disrupted cells. Later these small molecules are found in the 20, 30, and 50S particles. Evidently the ribosomes grow by the aggregation of RNA and protein molecules, perhaps to a preexistent core (Roberts *et al.*, 1959). As the process continues, the RNA units are joined to form larger entities of about a half and one million molecular weight. Concurrently, the 30S and 50S particles join to form the 70S particles which have been shown to be active in protein synthesis.

Dr. Aronson was a Carnegie Institution Fellow for 1959-60.

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Comment. This analysis has been superseded by that described in sections III.C.2 and III.C.3. The associations that take place under conditions of high Mg^{++} concentration among the various ribosomes and between ribosomes and their precursors are so complex as to obscure the true pattern of synthesis. Analysis of the various components revealed by the lower divalent ion concentration proved to be much more instructive. Brian J. McCarthy.

III.C.10 Hydrolysis of RNA by Lead Acetate

(Reprinted, by permission, from Comptes Rendus des Travaux du Laboratoire Carlsberg, vol. 32, no. 23, pp. 371-380, 1962.)

R. Britten¹

Molar lead acetate (pH 5.5) hydrolyzes RNA to nucleotides. The time for 50 percent hydrolysis at 25°C is about 20 minutes for free RNA and longer if the RNA is in the form of ribonucleoprotein.

1. INTRODUCTION

In the course of a search for an agent which would preserve the nuclei of *Tetrahymena pyriformis* by fixation it was discovered that concentrated solutions of lead salts preserved the gross appearance of the nuclei while permitting the RNA of the cell to go into solution. This led to the observation that the RNA was hydrolyzed to nucleotides while the DNA was resistant to hydrolysis. Since the hydrolysis of RNA by lead salts at room temperature had apparently not been previously observed and might be useful in studies of RNA structure, preliminary exploration of the process has been carried out.

2. METHODS

2.1 Preparation of RNA

The RNA of *Tetrahymena pyriformis*, *Salmonella typhimurium* and *Escherichia coli* was labeled by growing the cells in the presence of 2-¹⁴C-uracil. The radioactivity from 2-¹⁴C-uracil incorporated by these cells into macromolecules is found essentially only in the RNA as uridylic and cytidylic acids. A small fraction of the radioactivity appears in the cytidylic acid of the DNA. Nevertheless the hydrolysis of the RNA may be followed, without purification, simply by measuring the change of radioactivity of TCA (trichloro-

¹ Present address: Carnegie Institution of Washington, Department of Terrestrial Magnetism. This work was performed during a year's visit to the Carlsberg Laboratory and the University Institute of Microbiology, Copenhagen, Denmark. The author wishes to express his appreciation for generous hospitality and valuable discussion to Professor Heinz Holter and Professor Ole Maaløe.

acetic acid) precipitated samples. When cells were labeled with ^{32}P allowance was made for the labeling of cellular components other than RNA.

The labeled cells were washed in TSM buffer (tris hydroxy-amino methane, 0.01M; succinic acid, 0.004M to pH 7.4; magnesium acetate, 0.01M). For some experiments a sample of the whole cells was used. In other experiments the cell suspension in TSM was maintained at 100°C for 15 minutes ("boiled cells"). RNA was extracted by boiling in 1 M sodium acetate for 30 minutes followed by centrifugation. Smaller yields of RNA are achieved with sodium acetate than with sodium chloride. Small samples of supernatant were added to the lead acetate solution without removal of the sodium acetate.

Ribosomes were prepared from *Salmonella typhimurium* cells suspended in TSM and disintegrated in a modified Hughes press. Walls and unbroken cells were removed by centrifugation for 5 minutes at $40,000\times g$. The ribosomes were harvested by centrifugation for 180 minutes at $105,000\times g$. The TCA precipitable radioactivity in the supernatant was principally due to S-RNA and DNA. The pellet, which contained practically all of the ribosomes was resuspended in TSM and given a brief centrifugation to remove debris. The ribosomes were finally harvested by centrifugation for 180 minutes at $105,000\times g$ and resuspended in a small volume of TSM.

2.2 Hydrolysis conditions

Samples of RNA preparations in TSM were brought to 1 molar in lead acetate by adding 1.5 M lead acetate solution. The resulting suspension was incubated with occasional stirring at 25°C and samples (usually 0.050 ml) were diluted into 5 or 10 ml of 5 percent TCA. After 10 minutes at room temperature the TCA suspensions were filtered on collodion membrane filters (1). The filters were then dried and assayed for radioactivity. A great deal of experience with this technique has shown that macromolecules are completely harvested on the filters while low molecular weight compounds such as nucleotides are very little adsorbed. It is not known what size of oligonucleotides fail to be precipitated.

The lead acetate used was analytical grade $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2 \cdot 3\text{H}_2\text{O}$ and the concentration was established by the measurement of the specific gravity of the solution. The pH of the 1 M solution was about 5.5.

2.3. Chromatography of the hydrolysis products

Products of hydrolysis were examined by 1 dimensional paper chromatography after adsorption and elution from charcoal. The chromatographic solvent was isobutyric acid, NH_4OH (10 M), H_2O ; 66/1.2/33V/V/V. The products of hydrolysis were also separated on a Dowex-1 formate form column. For this purpose the 1 M lead acetate solution, containing ^{32}P labeled hydrolysis products, was diluted a factor of 20, unlabeled marker nucleotides were added, and the resulting solution poured over the column. Elution was then carried out with a gradient from 0 to 4 M formic acid.

3. RESULTS

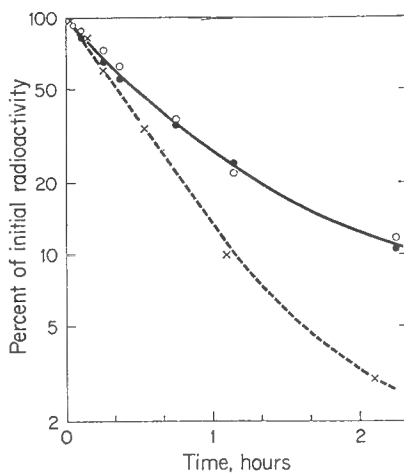
3.1. *Tetrahymena* RNA

Figure 1 shows the results (\circ) of an experiment in which washed C^{14} -uracil labeled *Tetrahymena* cells were suspended in 1 M lead acetate. The TCA precipitable radioactivity rapidly falls with an initial slope corresponding to a half life of about 20 minutes. It is clear that a large part of the RNA is rapidly hydrolyzed, however the lower slope at late times suggests that there may be a component in the RNA which is more resistant to hydrolysis. The fact that preliminary boiling of the cell suspension (before addition to the lead acetate) does not affect the rate of hydrolysis (\bullet) suggests that enzymes present in the cells have little effect on the reaction. This point will be more clearly demonstrated in section 3.3.

Only a small fraction of the RNA is extracted from *Tetrahymena* cells by boiling in 1 M sodium acetate. The data on figure 1, (\times) indicate that this fraction of the RNA is almost completely hydrolyzed within 2 hours.

The radioactivity of samples taken at very early times (less than one minute) have in

Fig. 1. Hydrolysis of *Tetrahymena* RNA at 25°C in 1 M lead acetate. RNA labeled by growth in the presence of ^{14}C -uracil. RNA assayed by measuring the TCA precipitable radioactivity harvested on membrane filters. Washed whole cells (\circ). Cells pretreated 15 minutes at 100° in TSM (\bullet). Extracted RNA (\times).



every case been equal to the TCA precipitable radioactivity of the preparations before addition of lead acetate. Therefore there is no instantaneous reduction of the TCA precipitability of the RNA due to the presence of the lead acetate.

3.2. *Salmonella* RNA

Figure 2 not only shows the hydrolysis of the RNA of *Salmonella typhimurium* but also indicates that the rate of hydrolysis depends

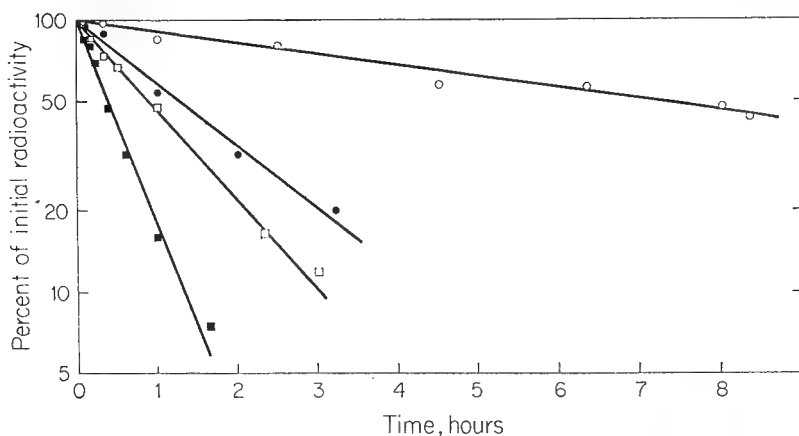


Fig. 2. Hydrolysis of *Salmonella* RNA at 25°C in 1 M lead acetate. Washed whole cells (○). Cells pretreated 15 minutes at 100°C in TSM (●). Ribosomes (□). Ribosomes pretreated 15 minutes at 100°C in TSM (■).

on the state of the RNA. The approximate half life times for hydrolysis in the four cases shown are: whole cells (○) 440 minutes; boiled cells (●) 80 minutes; ribosomes (□) 55 minutes; boiled ribosomes (■) 23 minutes. On figure 3 is shown the hydrolysis of three other preparations of *Salmonella* RNA and the approximate half periods are: S-RNA (Δ) 24 minutes; boiled S-RNA (▲) 21 minutes; RNA extracted by boiling 1 M sodium acetate (×) 18 minutes.

There is probably no significant difference between the rates of hydrolysis for these last three cases, the case of boiled ribosomes and the initial rate for *Tetrahymena* RNA. Thus 20 minutes can be taken as the half time for the hydrolysis of free RNA when measured in this way.

The apparent decrease in the rate of hydrolysis at late times shown on figure 3 is probably due to the presence of labeled DNA in these fractions. The DNA is not hydrolyzed (see section 3.4) and the amount apparently present in the S-RNA fraction is

reasonable although no quantitative estimate of its degree of labeling and relative yield can be made.

3.3. Effect of Ribonuclease

It might be suggested that the effect of lead acetate is to activate latent ribonuclease (2), (3). However the fact that boiling either has no effect or increases the rate of hydrolysis indicates that this is not so. The data presented in figure 4 show that ribonuclease originally present in the RNA preparations has no significant effect under the conditions of hydrolysis. In the first place the boiled ribosome preparation shows no hydrolysis in the absence of lead acetate even though ribonuclease, if it

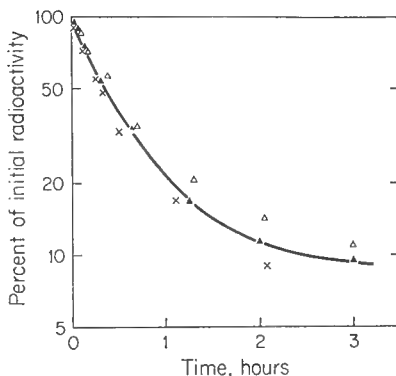


Fig. 3. Hydrolysis of *Salmonella* RNA at 25°C in 1 M lead acetate. S-RNA (Δ). S-RNA pretreated 15 minutes at 100°C in TSM (▲). Extracted RNA (x).

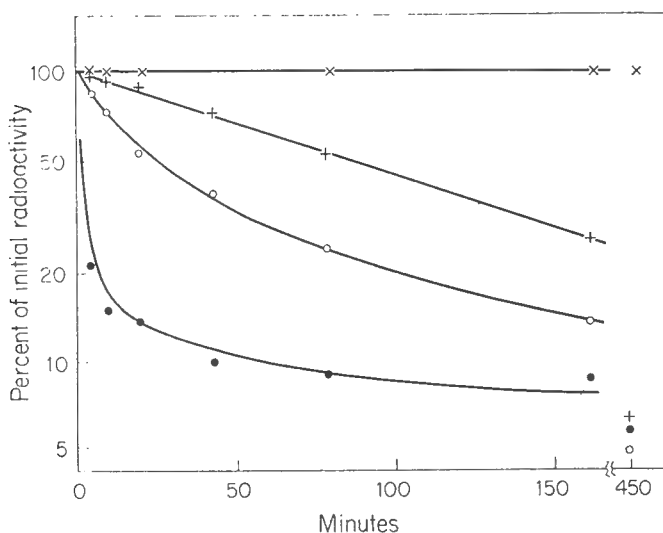


Fig. 4. Inhibition of RNAase by 0.1 M lead acetate. TCA precipitable radioactivity collected on membrane filters as a function of time of incubation at 26°C in the following solutions: (+) 0.1 M lead acetate; (●) 5 micrograms per ml pancreatic ribonuclease; (o) 5 micrograms per ml ribonuclease, at 0.1 M lead acetate; (x) control, suspended in TSM. RNA was prepared from ¹⁴C-uracil labeled *Salmonella typhimurium* ribosomes by boiling 20 minutes in TSM.

survived the boiling would not be expected to be still latent. In the second place the lower pair of curves show that 0.1 M lead acetate strongly inhibits the action of beef ribonuclease.

3.4. *Lack of hydrolysis of DNA*

It appears that DNA is hydrolyzed very slowly if at all by molar lead acetate at 25°C. When ^{14}C -thymine labeled *Tetrahymena* cells were suspended in molar lead acetate there was no significant change in the amount of TCA precipitable radioactivity over 24 hours. As an additional verification T-4 bacteriophage were suspended in molar lead acetate. Over several days no significant change was observed in the amount of ultraviolet absorbing material (DNA) which could be extracted from TCA precipitated samples with hot perchloric acid.

The absence of hydrolysis of DNA is also indicated by the apparent preservation of the morphological structure of *Tetrahymena* nuclei in molar lead acetate. When *Tetrahymena* are stained with acridine orange (12) and observed in the fluorescence microscope the macronucleus shows a bright green fluorescence while a number of bright orange RNA-containing granules are distributed throughout most of the cytoplasm. When these stained cells are immersed in molar lead acetate the orange fluorescence rapidly disappears while the green fluorescence of the macronucleus is relatively little changed.

3.5. *The products of hydrolysis*

The products of hydrolysis by lead acetate were first examined by paper chromatography. For this purpose whole ^{14}C -uracil labeled *Salmonella typhimurium* cells were boiled for 20 minutes, centrifuged, washed and suspended in 1 M lead acetate for 20 hours at 25°C. On centrifugation and desalting by absorption on charcoal half of the labeled material was lost. 90 percent of the recovered radioactivity ran coincident with carrier 2' and 3' uridylic and cytidylic acids.

In order to measure the yield of nucleotides in comparison with other possible hydrolysis products ribosomes were prepared (as described in 2.1) from *Salmonella* grown overnight in the presence of ^{32}P - phosphate. The ribosome suspension was boiled for 20 minutes, cooled, brought to 1 M in lead acetate and incubated for 6-1/2 hours at 25°C. The hydrolysis products were analyzed on Dowex 1, as described in section 2.3, and the results are shown on figure 5.

The suspension after hydrolysis was diluted a factor of 20 in water and carrier 5' cytidylic and 2'-3' adenylic acids were added. The radioactivity was completely adsorbed on the column. 84 percent of the radioactivity appears in four peaks corresponding to the four 2'-3' nucleotides. The remaining 16 percent of the radioactivity was not eluted from the column when the run was terminated just after the uridylic acid had been eluted. Less than 1 percent

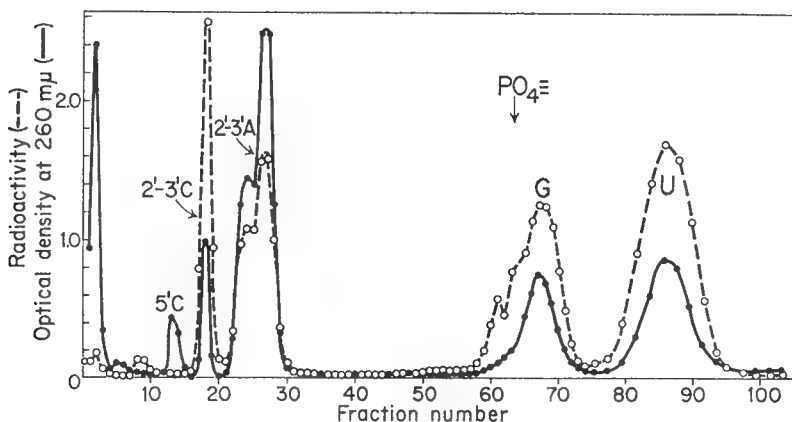


Fig. 5. Chromatography of hydrolysis products on Dowex 1. 0-4 M formic acid gradient elution. Boiled ^{32}P labeled *Salmonella* ribosomes hydrolyzed for $6\frac{1}{2}$ hours at 25°C in 1 M lead acetate. Unlabeled 5'-cytidylic acid and 2'-3' adenylic acid markers added. C, cytidylic acid; A, adenylic acid; G, guanylic acid; U, uridylic acid. (---○--- ^{32}P radioactivity. (—●— optical density at 260 mμ).

of the radioactivity occurs outside of the four peak regions. The 5' cytidylic carrier was completely resolved from the 2'-3' cytidylic peak and no radioactivity was eluted with it.

Orthophosphate is eluted at the leading edge of the guanylic acid peak. In this analysis there is in fact a somewhat higher specific radioactivity at the leading edge of the guanylic acid peak. If this were due to orthophosphate the amount present is less than 5 percent of the radioactivity appearing in the four nucleotide peaks. There is perhaps an equivalent quantity of ultraviolet absorbing material eluted in the first 3 fractions where bases and nucleosides would be expected to appear, however a major part of this may be due to the lead acetate solution.

It is clear that molar lead acetate at 25°C very nearly completely hydrolyzes RNA to nucleotides in 6 hours or less. The products

are not 5' nucleotides and apparently are mixtures of 2' and 3' nucleotides.

3.6 Hydrolysis of the ribosome precursors

In *E. coli* there exist fractions of the RNA which are very rapidly labeled with ^{14}C -uracil. (4, 5, 6). The kinetics of labeling of these fractions and their behavior in sedimentation analysis show

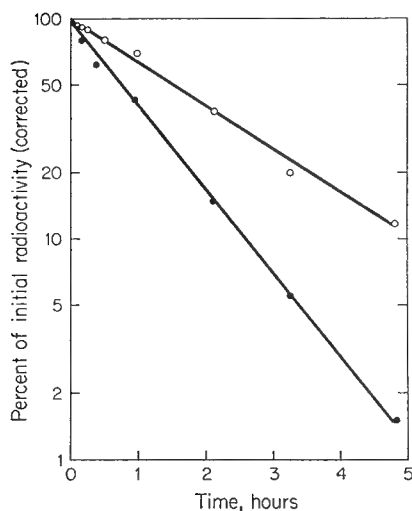


Fig. 6. Hydrolysis of *E. coli* ribosomal precursor RNA at 25°C in 1 M lead acetate. See text for details. (●) ^{14}C radioactivity as a measure of precursor RNA, corrected for estimated radioactivity in DNA by subtracting 2 percent of the original total ^{14}C radioactivity. (○) ^{32}P radioactivity as a measure of total RNA, corrected for estimated radioactivity in compounds other than RNA by subtracting 17 percent of the original total ^{32}P radioactivity.

that they are precursors to ribosomal RNA (7). Since these studies indicate that the precursors consist of ribonucleoprotein of low protein content a measurement of their rate of hydrolysis by lead acetate was carried out, and the results are shown in figure 6.

In order to be able to measure the hydrolysis of the total RNA of the cell and the precursor RNA together in the same experiment, *E. coli* cells were grown for 3 generations in the presence of ^{32}P -phosphate and then supplied 2- ^{14}C -uracil for 2 minutes. Incorporation was stopped by chilling the culture to 0°C and the cells were washed and broken as described in (7). The ^{14}C - and ^{32}P -radioactivities were simultaneously assayed using the Tri-Carb liquid scintillation counter.

The experiments described in (7) show that the first stage precursor in the complex process of ribosome synthesis contains about 90 per cent of the ^{14}C -radioactivity after 2 minutes exposure to ^{14}C -uracil. Figure 6 shows that this early labeled RNA is hydrolyzed at about twice the rate of the average RNA of the cell. The rate is, however, not so great as that for free RNA.

4. DISCUSSION

The hydrolysis of RNA by lead acetate has a striking similarity to the hydrolysis by alkali. The rates of the two processes are comparable and the products (2'-3' nucleotides) apparently identical. Further, DNA is resistant to hydrolysis by both reagents. No studies have been made which suggest the chemical mechanism involved but it seems likely that an examination of the chemistry of the hydrolysis should lead to further understanding of the structure of RNA. The kinetics of the reaction appear to be simple. In several cases (figures 2 and 6) no curvature is observed on the semi-log plots. In other cases where curvature is observed the substrate may very well contain several components. It is apparently the lead that is involved since sodium acetate and calcium acetate give no measurable hydrolysis while lead nitrate is effective.

When concentrated solutions of RNA are brought to 1 M in lead acetate a heavy precipitate is first formed which redissolves completely between 20 minutes and 1 hour. Interestingly, Stevens and Duggan (8) and Sinsheimer (9) have observed that single stranded or denatured DNA is precipitated by dilute lead salts while native double stranded DNA is not.

The fact that RNA present in bacterial ribosomes is hydrolyzed at one third of the rate of free RNA suggests that there are chemical bonds to the phosphorus or ribose of the RNA in the nucleoprotein which inhibit the action of the lead ions. A few measurements show that the rate of hydrolysis is considerably greater at 37°C than at 25°C. However, the difference in the rates of hydrolysis of free RNA and nucleoprotein is diminished. A further study of these phenomena should lead to some insight into the structure of the nucleoprotein.

The absence of this effect in *Tetrahymena*, although other evidence indicates that the RNA is principally ribosomal (10), suggests that the organization of *Tetrahymena* ribosomes may be different from the bacterial ribosomes. That some difference exists is also indicated by the observation that *Tetrahymena* ribosomes do not go into solution when the cells are passed through a modified Hughes press—a method that will bring bacterial ribosomes almost entirely into solution.

A number of experiments (11) suggest that bacterial ribosomes do not exist in the cell in the form observed after the cell is broken open. When undamaged bacterial cells are suspended in molar lead acetate their RNA is hydrolyzed at a much slower rate than free or ribosome bound RNA. Since the TCA precipitable RNA falls

exponentially over 20 hours (down to 10 percent of the original quantity) without any sign of increasing rate, the slow hydrolysis is presumably not due to a slow penetration of lead ions into the cell. Although there are obvious alternative explanations, this result may imply that the RNA in the whole cell is bound in such a way as to protect it from the lead ions, and in a way that differs from the protection observed in isolated ribosomes.

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III.C.11 The Effects of Magnesium Starvation on the Ribosome Content of Escherichia coli

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SUMMARY

Escherichia coli cells incubated for long periods in a growth medium complete except for magnesium suffer loss of their ribosomes. There is no concomitant loss in viability. Cell extracts prepared after various times show first a loss of 70-S and 100-S ribosomes, and after some 24 h only 5 % of the original ribosomes remain. Re-addition of magnesium ions to the medium causes renewed growth at a steadily increasing rate with ribosomes increasing exponentially. The rate of protein synthesis is proportional to the ribosome content.

Ribosomes are degraded during starvation to structures of low S number but the RNA is not broken down to small molecules nor is it re-utilized for ribosome synthesis on renewed growth.

INTRODUCTION

The importance of Mg^{2+} to the structural integrity of ribosomal particles has been established for a variety of ribosomal preparations¹⁻⁵. Such studies have shown that as the magnesium concentration of the *in vitro* preparation is reduced the larger ribosomes first dissociate to smaller subunits and later disintegrate. Studies of pea-seedling ribosomes by EDELMAN *et al.*⁶ have shown that a Mg:P molar ratio of 0.3 is necessary for the intactness of the larger ribosomes.

The present study was undertaken in order to elucidate any parallel changes which might occur actually within the bacterial cell during deprivation of magnesium *in vivo*.

MATERIALS AND METHODS

Cells of *E. coli* B were grown in a glucose-salts medium consisting per litre of: NH_4Cl , 2 g; Na_2HPO_4 , 6 g; KH_2PO_4 , 3 g; $NaCl$, 3 g; Na_2SO_4 , 0.1 g; $MgCl_2$, 0.01 g and glucose, 10 g in which the mean generation time is 50-55 min at 37°.

The medium used for magnesium starvation was identical except for the lack of $MgCl_2$.

When the uptake of ^{35}S was being followed as a measure of the rate of protein synthesis, the concentration of Na_2SO_4 was reduced to 0.01 g/l.

Abbreviations: TCA, trichloroacetic acid; s-RNA, soluble RNA.

Cells were randomly labeled with ^{32}P by growing for several generations in the normal medium to which 1 mC/l of $[^{32}\text{P}]\text{PO}_4$ had been added.

The increase in cell mass of a culture was followed by observing the absorbancy at 650 $\text{m}\mu$. Plate counts were made to follow cell viability.

Cell-free extracts were prepared by washing the cells three times in a Tris 0.01 M , succinic acid 0.004 M , magnesium acetate 0.01 M , pH 7.4 buffer, and disrupting the resulting cell suspension in a French pressure cell at 10 000–15 000 pounds/in.². At least 95 % of the cells were broken.

The cell extracts were analyzed for ribosome content in one of two ways. Total ribosome content was determined by first removing the cell walls from an aliquot of cell juice by means of a 10-min centrifugation in the Spinco Model L Ultracentrifuge at 25 000 rev./min and then centrifuging the supernatant for 4 h at 40 000 rev./min. The pellet contained about 95 % of the ribosomes and the quantity could be estimated from the absorption at 260 $\text{m}\mu$ or the ^{32}P label. In order to eliminate systematic errors, ribosome contents were always expressed as a percentage of exponentially growing cells estimated at the same time.

In other cases the comparison made was an internal one. ^{32}P -labeled exponential-cell extract was added to the sample and the ribosome content determined by isotopic dilution. In cases where the sample itself was labeled the carrier consisted of a large quantity of exponential-cell extract having more than twenty times the ultraviolet absorption of the sample. Ribosome contents could be expressed again as a percentage of that of exponential cells by measurement of the ratio of ^{32}P to absorbancy at 260 $\text{m}\mu$ in the pellet and the supernatant.

Other determinations of ribosome contents were made with the aid of the Spinco Model E Ultracentrifuge. Schlieren photographs were made of the cell extracts and the ribosome content assessed from the areas under the peaks compared with the area of the soluble-protein peak. In these determinations, also, the results were expressed as a percentage of the ribosome content of exponentially growing cells.

The DEAE-cellulose used for chromatography of cell extracts had a capacity of 1.1 mequiv/g and was purchased from the Eastman Chemical Co.

RESULTS

General effects of magnesium starvation

Preliminary experiments showed that the effects of magnesium starvation on a culture of cells were very dependent on the initial state of the culture. Cells in a resting state washed free of magnesium and starved in a magnesium-free medium showed a much less rapid rate of loss of ribosomes than exponentially growing cells similarly treated. To obtain consistent results, therefore, cells were always followed through three generations of exponential growth before being starved. Washing the cells three times with a magnesium-free medium before starvation insured repeatable results. Starvation was carried out at 37° in a medium complete except for magnesium.

Extensive periods of starvation do not have any appreciable effects on cell viability. In most cases, the absorbancy of a culture of cells increases by some 50 % soon after transferring to a magnesium-free medium (Fig. 1).

The number of viable cells increases during the first few hours and then remains constant for at least 40 h (Fig. 1). After 3 h of starvation, when increases in absorb-

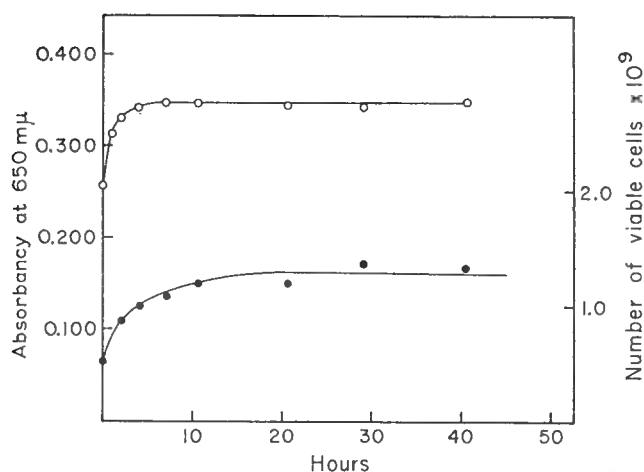


Fig. 1. Increase in absorbance at 650 mμ, ○-○, and number of viable cells, ●-●, of a culture of *E. coli* washed free of external magnesium and resuspended in a magnesium-free medium.

ancy and number of cells had ceased, rates of protein and DNA synthesis fell to less than 3 % of that of exponential cells.

The ribosome content as observed by means of the Schlieren optical system of the Spinco Model E Ultracentrifuge does, however, show some dramatic changes. The left-hand column of Fig. 2 contains pictures of cell extracts made after increasing periods of magnesium starvation. Cells were washed three times with Tris buffer containing magnesium at $10^{-2} M$, so that all extracts were made in the presence of the same $10^{-2} M$ magnesium concentration thus eliminating any *in vitro* effects of magnesium ions on ribosome stability. As can be seen from Fig. 2, the first effect is the loss of the larger 100-S and 70-S ribosomes. After 6 h of starvation only a small peak of 70-S remains visible. More extensive starvation eliminates both 50-S and 30-S ribosomes until by 20 h no ribosome peaks remained. At this point isotopic dilution with ^{32}P -labeled exponential-cell extract showed the ribosome content to be only 5 % of that of exponential cells.

Growth of magnesium-starved cells upon re-addition of magnesium

Restoration of magnesium to a culture of cells starved for 24 h produces measurable growth within 0.5 h. The overall growth rate is very low initially but increases continuously. Well-starved cells take 5–6 h to resume exponential growth with the normal generation time 50–60 min (Fig. 3). This type of growth curve is not a result of the multiplication of a small fraction of the starved cells since they are all viable (Fig. 1).

Although the increase in total cell mass as measured by absorbance shows this retardation, the number of ribosomes in the culture increase in an interesting manner. The increase in the total ribosomal material in an aliquot of culture is plotted in Fig. 4. Soon after the addition of magnesium to the culture the ribosomal material increases exponentially with a generation time of 52 min. Thus ribosomes are being reproduced at the optimum exponential rate even though the total cell mass is not increasing exponentially. The slow rate of increase of the absorbance must mean

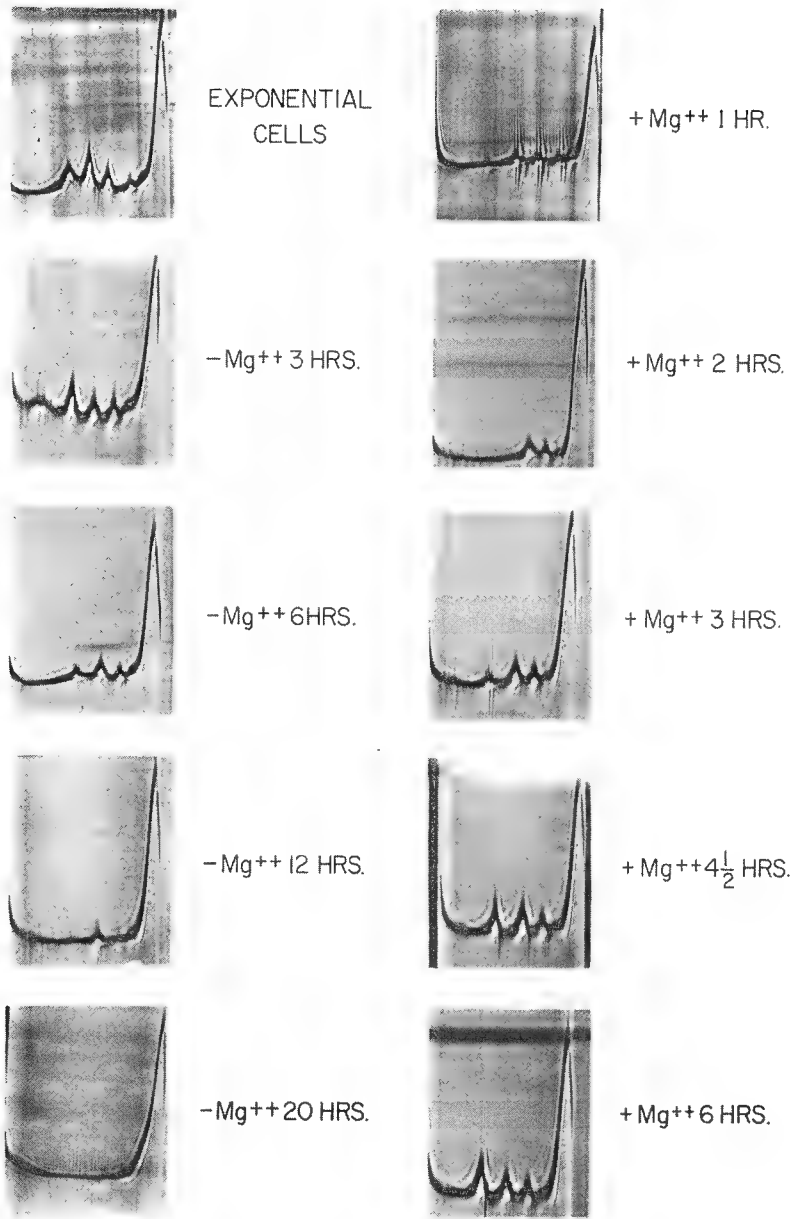


Fig. 2. Schlieren photographs of samples of extract made from cells during magnesium starvation and subsequent re-addition of magnesium. Pictures 8 min after reaching 50 740 rev./min. Bar angles varied between 30° and 45° so as to equalize the height of the soluble-protein peak in extracts of different concentrations. Sedimentation from right to left. The sedimentation coefficients of the four peaks in the first frame are approx. 95 S, 70 S, 50 S and 30 S from left to right. The left-hand column represents a series of extracts taken from cells after various periods of magnesium starvation. The single peak remaining in frame 4 is 50 S. The right hand column represents a series of extracts taken after the re-addition of magnesium at 20 h.

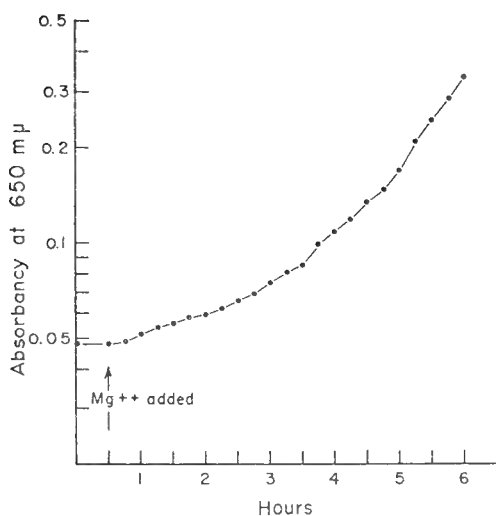


Fig. 3. The increase in absorbance at 650 $m\mu$ of a culture of *E. coli* starved for magnesium for 24 h before the re-addition of magnesium.

that protein synthesis is not optimal. It is as though an individual cell must restore its ribosome content to normal before protein synthesis and ribosome synthesis are again balanced and exponential growth can be resumed. Calculations from the data of Fig. 4 and direct measurements show that the ribosome content per cell is restored to that of normal exponentially growing cells between 5 and 6 h after re-addition of magnesium. This is approximately the time at which their growth curve (Fig. 3) becomes exponential and the generation time is again 50–55 min.

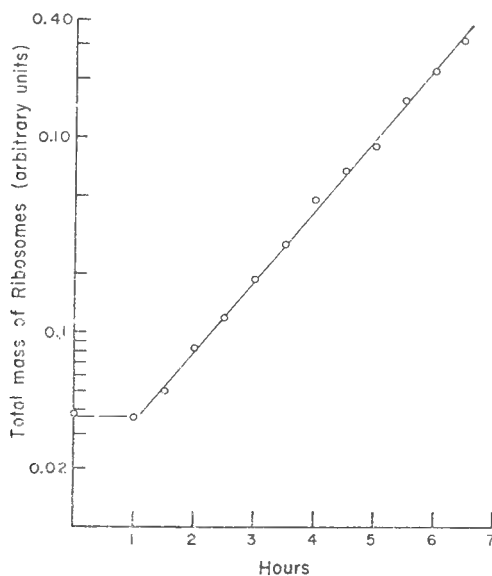


Fig. 4. The increase in the quantity of ribosomal material of a culture of cells starved for magnesium for 24 h before the re-addition of magnesium. Ribosomal material was determined as described in the text and expressed in terms of the concentration per ml of culture. Magnesium added at 30 min.

Observations of the appearance of the various sizes of ribosomes were made with the analytical ultracentrifuge. Pictures of five extracts taken after the re-addition of magnesium are shown in Fig. 2 (right-hand column). The 30-S and 50-S ribosomes are the first to appear and these build up to a fairly high level during the first 2–3 h before the appearance of many 70-S ribosomes. By 6 h the ribosome pattern has returned almost to normal.

Protein synthesis in magnesium-starved cells

As already observed, RNA synthesis is rapidly renewed at an exponential rate after the re-addition of magnesium. Protein synthesis also begins very slowly and the rate of protein synthesis as measured by the rate of uptake of ^{35}S per mg of cells increases steadily for the first 5 h to reach a constant value. Through most of the recovery period the rate of protein synthesis is approximately proportional to the ribosome content of the cells. Ribosome content is expressed as a percentage of that of exponential cells (Fig. 5). The curvature apparent at low ribosome contents suggests that the efficiency of protein synthesis per ribosomal particle is low during the

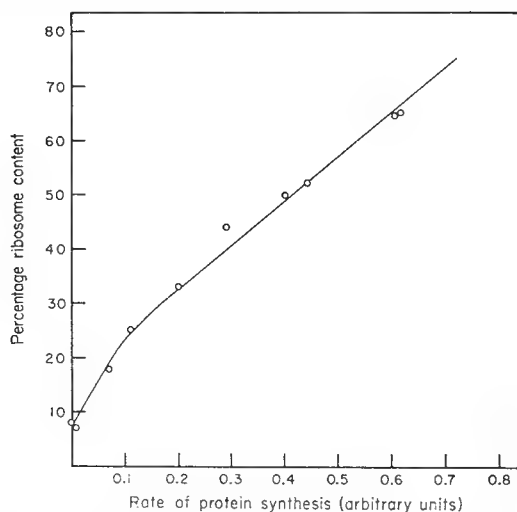


Fig. 5. The relationship between ribosome content and the rate of protein synthesis in cells to which magnesium had been added back after 24 h starvation. The ribosome content is expressed as a percentage of that of exponentially growing cells and the rate of protein synthesis in arbitrary units in terms of the ^{35}S radioactivity taken up into protein per min per mg of cells.

early stages of recovery from magnesium starvation. If it is really the 70-S ribosomes which carry out most of the protein synthesis as suggested by the experiments of McQUILLEN *et al.*⁷, the curvature may be ascribed to the fact that most of the ribosomes synthesized during the first 2 h exist as 50-S and 30-S. It is conceivable that these are able to synthesize protein only at a rate lower than that of the 70-S.

Moreover, during the first 2 or 3 h a very high proportion of the protein synthesized is ribosomal. When an extract was made from cells given ^{35}S for the first 2 h of recovery from magnesium starvation, 89 % of the radioactivity of the cell-wall-free supernatant was collected with ribosomes by means of centrifugation for 3 h at 40 000 rev./min. This can be compared with 49 % collected from a control culture of expo-

nential cells. Such a crude pellet is, of course, by no means all ribosomes for ribosomal protein represents only about 20 % of the total. The very high figure obtained does, however, indicate preferential synthesis of ribosomal protein during the recovery of cells from magnesium starvation. No more exact measurements were attempted since it is clear that the synthesis of complete ribosomes does occur preferentially to restore the ribosome content of the individual cells to normal and that this must require preferential synthesis of ribosomal protein over soluble protein. Measurements of the synthesis of β -galactosidase after magnesium starvation by ARONSON AND DUERKSEN⁸ showed no increase during the first 2 h of recovery. This was true for either constitutive or induced synthesis of the enzyme.

Fate of ribosomal RNA after magnesium starvation

After extensive magnesium starvation, peaks of ribosomal material may be completely absent from a cell extract (Fig. 2). On the other hand, there is no appreciable loss of RNA from the cell as TCA-soluble material. Degradation of ribosomes thus appears to proceed only as far as a macro-molecular structure of a size less than would be observable in the analytical ultracentrifuge. This suggested the possibility that magnesium starvation merely dissociated the RNA and protein moieties of ribosomes and that this could be reversed on renewed growth. Experiments were therefore designed to determine whether any of the RNA of ribosomes degraded by magnesium starvation was re-incorporated into ribosomes subsequently appearing.

Chromatography of the cell extract on DEAE-cellulose proved to be a technique suited to this purpose. Elution from DEAE-cellulose with a linear sodium chloride gradient produces resolution of nucleic acid-containing compounds among three regions⁹. The first region, at 0.4 *M* NaCl contains the bulk of the ribonucleoprotein and the second, at 0.5 *M* NaCl contains soluble-RNA largely unresolved from DNA. One of the ribosome precursors also appears in this region. The third peak at 0.6 *M* NaCl contains the primary ribosome precursor. So far as ribonucleoproteins are concerned the resolution among the three regions reflects a progressively lower protein: RNA ratio as a higher salt concentration is required for elution.

Fig. 6a shows the elution diagram on an extract made from cells labeled for three generations in ³²P followed by 0.5 h in ³¹P medium to remove the pool of soluble ³²P-compounds. Another sample of the same cells was starved in a magnesium-free medium containing unlabeled phosphorus for 12 h (Fig. 6b). This results in the transfer of ultraviolet absorption and ³²P label from the first at 0.4 *M* salt to the third peak at 0.6 *M* salt. The decrease in the counts appearing in the first peak agreed with the reduction of ribosome content to 20 % of normal. The middle peak consisting mostly of s-RNA remains constant.

After 18 h of starvation magnesium was restored and growth allowed for a further 3 h. A third extract was then prepared and chromatographed (Fig. 6c). During this period the total ribosome content increased by a factor of 5. These newly made ribosomes cause an increase in the ultraviolet absorption of the first peak. The fact that the specific activity falls by the same factor as the ribosome increase, and that none of the counts were transferred from the 0.6 *M* NaCl peak back to the 0.4 *M* NaCl peak, proves that renewed ribosome synthesis occurred without re-utilization of the degenerate ribosomal material produced by magnesium starvation.

The chromatographic behavior of the degenerate ribosomal material suggests

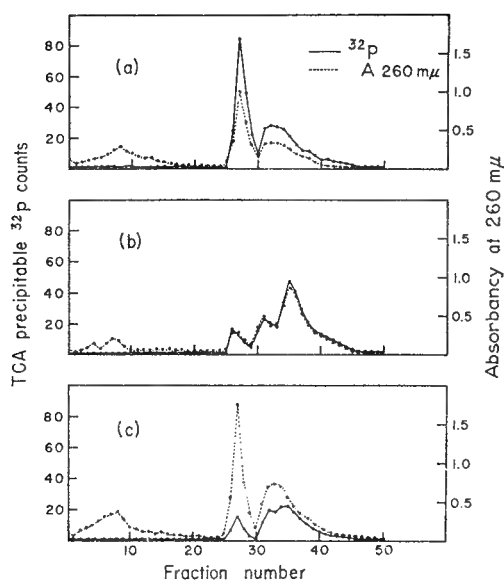


Fig. 6. Chromatography on a DEAE-cellulose column of three cell extracts. Elution with a linear sodium chloride gradient from zero to $1.0\text{ }M$ (0.004 mole/ml) in Tris $0.01\text{ }M$, succinic acid $0.004\text{ }M$, magnesium acetate $0.01\text{ }M$, pH 7.4. Fractions collected 3.5 ml . (a) Exponentially growing cells labeled for three generations with ^{32}P followed by 0.5 h in ^{31}P ; (b) Cells of (a) starved of magnesium for 12 h ; (c) Cells of (b) 3 h after re-addition of magnesium. Salt concentration at peaks left to right $0.40\text{ }M$, $0.51\text{ }M$, $0.60\text{ }M$.

that much of the protein has been dissociated⁹. Elution at $0.6\text{ }M$ is characteristic of ribosomal RNA in precursor stages when it associated with only small quantities of protein. It does not, however, appear to be completely free of protein since phenol extraction of an extract of magnesium-starved cells results in free ribosomal RNA which elutes at $0.7\text{--}0.8\text{ }M\text{ NaCl}$ (see ref. 10).

To summarize, magnesium starvation results in dissociation of most of the ribosomal protein from ribosomal RNA. The resulting material has an S number of less than 20, as shown by Schlieren photographs, or the sucrose-gradient technique of BRITTON AND ROBERTS¹¹. Extraction with phenol results in RNA with an average sedimentation coefficient of about 4 (see ref. 12). The degenerate RNA is degraded only slowly to nucleotides and it is not re-utilized for ribosome synthesis.

DISCUSSION

The degradation of ribosomes in the living cell as a result of magnesium deprivation is not unexpected. Isolated 70-S and 100-S ribosomes from *E. coli* dissociate to 50-S and 30-S at magnesium concentrations of less than $2 \cdot 10^{-3}\text{ }M$. At concentrations of less than about $3 \cdot 10^{-5}\text{ }M$ dissociation of the protein and RNA moieties is accompanied by the liberation of the latent RNAase¹³ and subsequent digestion of the RNA. A similar sequence of events is to be expected in the living cell.

On the other hand, it is perhaps surprising that the bacterial cells can survive the almost complete loss of these important elements in their functional architecture amounting to some 25 % of their mass. The changes in ribosome content commence with the loss of the larger particles and continue with the apparent dissociation of the

protein from the ribosomal RNA. However this disorganization of ribosomal structure does not liberate ribonuclease to any extent. This is evident from the fact that the RNA remains essentially TCA-precipitable even when ribosome peaks are no longer visible in the analytical ultracentrifuge.

The recovery of cells from exhaustive magnesium starvation is in many ways analogous to the shift-up experiments of NEIDHART AND MAGASANIK¹⁴ and KJELDGAARD¹⁵. In both cases the optimum rate of protein synthesis of which the cells are capable in the new medium demands a much higher ribosome content. In magnesium-starved cells the resultant preferential synthesis of ribosomes is dramatically emphasized. In the initial stages of recovery as much as three quarters of the protein synthesized may be ribosomal. During this increase in the number of ribosomes per cell the rate of protein synthesis is approximately proportional to the number of ribosomes over a factor of six.

The kinetics of the increase in the number of ribosomes in the recovery from magnesium starvation suggest that the rate of synthesis is limited by the number of ribosomes present. This exponential increase of ribosomes under conditions where the total cell mass is certainly not increasing at optimum rate indicates that the synthesis of ribosomes may be in some way autotrophic. This could imply that ribosome synthesis takes place on ribosome templates by self-duplication of the RNA. It is clear from kinetic studies of ribosome synthesis⁹ that the first stage involves the production of ribosomal RNA, largely free of protein, but loosely associated with ribosomes and that ribosomal protein is added subsequently.

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III.C.12 Alternative Codes and Templates

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The problem of deducing the "biological code" from the composition of various natural RNA's and their product proteins is difficult if not impossible. The converse problem of calculating the composition of a template from the known composition of its protein product according to an assumed code requires only a few minutes. Such a calculation to determine the composition of the template RNA which would be required to direct the synthesis of bacterial proteins according to recently published triplet codes¹⁻⁷ is given in Table 1. This hypothetical RNA has a high uracil content (45%) and is quite unlike any natural RNA.

On the other hand, the triplet code can be converted to a doublet by discarding the U common to all of the code words. A calculation of the hypothetical template RNA on this basis shows a composition which resembles ribosomal RNA. Table 2 shows a comparison of the template material (calculated according to the doublet code) with observed RNA's of several bacteria in which the proteins⁸ and RNA⁹ have been carefully analyzed. In all cases, the calculated template is similar to the 50S ribosomal RNA. No exact agreement can be expected since the efficiencies of different templates may vary.

The doublet code obtained by discarding the U common to the triplet words is shown in Table 3. The order is based on the amino acid replacement data.^{6, 7} The complete use of all the possible doublet symbols is perhaps significant. In writing out this code, the apparent degeneracies were omitted. These can be attributed to errors in the attachment of certain amino acids to the S-RNA which acts as carrier in the cell-free system.¹⁰ Leucine, for example, might be attached to the carriers for valine and isoleucine. These three amino acids also confuse the entry mechanism of the cell.¹¹⁻¹³

In several respects, the two codes are equivalent. They predict the same incorporation of amino acids relative to phenylalanine, as the ratio XUU/UUU is the same as XU/UU. They are equally satisfactory in fitting the amino acid replacement data.

They differ markedly in predicting the stimulation of phenylalanine incorporation to be expected from synthetic polymers of reduced U content. The doublet code has the possible theoretical advantages that it uses fewer letters, contains no "nonsense," and agrees with the correlations found by Sueoka.⁸

The three-letter code has the serious failing that it predicts template RNA unlike any natural nucleic acid yet observed. This difficulty will be resolved if it turns out that the present symbols are only a small and little-used part of a highly degenerate code which includes many symbols lacking U.

The doublet code provides no reason that certain synthetic polymers (poly A, poly C, poly G, poly AC, poly GC, poly AG) do not act as templates. All of these

TABLE 1
CALCULATION OF HYPOTHETICAL TEMPLATE

	Proportions in <i>E. coli</i> proteins*	Code	Common U	Proportion of Bases Expected in Template			
				U	G	A	C
Ala	14.7	UCG	14.7		14.7		14.7
Arg	7.0	UCG	7.0		7.0		7.0
Asp	14.4	UAG	14.4		14.4	14.4	
Cys	0.9	UUG	0.9	0.9	0.9		
Glu	15.6	UAG	15.6		15.6	15.6	
Gly	12.0	UGG	12.0		12.0		
His	2.9	UAC	2.9			2.9	2.9
Ileu	7.5	UUA	7.5	7.5		7.5	
Leu	12.4	UUC	12.4	12.4			12.4
Lys	8.5	UAA	8.5			8.5	
Met	4.3	UAG	4.3		4.3	4.3	
Phe	4.8	UUU	4.8	4.8			
Pro	5.8	UCC	5.8				5.8
Ser	6.5	UUC	6.5	6.5			6.5
Thr	7.8	UAC	7.8			7.8	7.8
Try	—	UGG	—				
Tyr	3.9	UUA	3.9	3.9		3.9	
Val	10.0	UUG	10.0	10.0	10.0		
				1390.	508.	909.	734.
Composition of template (including common U)				45.1	21.8	17.6	15.1
Composition of template (excluding common U)				18.3	32.7	26.4	22.6
Composition of <i>E. coli</i> 50S ribosomal RNA†				19.6	33.5	25.4	21.5

* Data of Sueoka.⁸

† Data of Midgley.⁹

possible combinations should provide sites. In this respect, it has the same flaw as the triplet code since symbols lacking U have not been detected.^{6, 7} In both cases, the difficulty can be attributed to special properties of polyphenylalanine or poly U which are essential in cell-free systems.

The doublet has the serious failing that it provides only 16 combinations. Perhaps asparagine and glutamine could be converted from aspartic acid and glutamic acid after incorporation, but it is difficult to extend this reasoning to the other ambiguities, methionine and tryptophan. An unlikely possibility is that unusual bases or missing bases provide a few needed code words. A more plausible escape from this dilemma lies in a mixed code which includes a few three-letter symbols. If, for example, the combinations AA and GG indicated the start of a three-letter word, there would be a sufficiency of combinations. The mixed code would also provide a mechanism which could occasionally produce the results of Crick *et al.*¹⁴ Another interpretation is that the cell can distinguish two kinds of purine pairs (e.g. parallel and anti-parallel).

The finding that the hypothetical templates calculated according to the doublet code resemble ribosomal RNA raises another question. In the growing cell, newly formed RNA can be distinguished by chromatography or sedimentation.^{15, 16} Roughly three per cent of the RNA is in this form, one per cent being DNA-like in composition and two per cent being ribosome-like.⁹ Experiments with cell free systems suggest that the newly formed material is the most likely template,¹ but there is no evidence as to which component is active. In Table 2, the predicted template material shows a slight correlation with changes in the DNA composition but the correlation is less than would be expected if the DNA-like component were fully active as a protein-forming template. Thus, the ribosome-like component of

TABLE 2
COMPARISON OF RNA'S

Organism	GC content of DNA (%)	Type of RNA	Mole Per cent			
			U	G	A	C
<i>B. subtilis</i>	42	Template*	18.5	32.1	27.5	21.8
		50S†	19.3	32.0	26.5	22.5
		Newly synthesized†	23.7	27.3	25.5	23.5
<i>E. coli</i>	50	Template	18.3	32.7	26.4	22.6
		50S	19.6	33.5	25.4	21.5
		Newly synthesized	22.6	29.5	25.0	22.9
<i>A. aerogenes</i>	57	Template	18.1	33.4	25.2	23.2
		50S	21.2	31.2	25.6	22.0
		Newly synthesized	21.5	30.3	24.8	23.4
<i>Ps. aeruginosa</i>	65	Template	17.4	33.9	24.5	24.1
		50S	21.3	31.2	26.3	21.2
		Newly synthesized	20.5	31.9	21.4	26.2

* Hypothetical template calculated according to doublet code from amino acid analyses of Sueoka.⁸
† Observed RNA compositions (Midgley⁹).

TABLE 3
A DOUBLET CODE

First letter	Second Letter				
	U	C	G	A	
	phe	ser	cys	tyr	
	leu	pro	arg	his	
	val	ala	try	glu	
			gly	glu N	
	ileu	thr	asp	lys	
			met	asp N	

Letters based on amino acid incorporation; order based on amino acid replacements.

the newly formed RNA would appear to act as the template for most of the cell's proteins.

This view cannot be ruled out at present. During one generation, the growing cell might make two RNA copies of all its DNA, thereby providing the observed rate of synthesis of the DNA-like RNA. Such a rate could be characteristic of the production of templates for uninduced (or repressed) enzymes or for RNA copies of nonstructural genes. This DNA-like RNA is degraded and reutilized to form stable nucleic acids,¹⁷ possibly after serving as template for a small part of the protein.

At the same time, a limited group of DNA sites (perhaps 1,000 of a possible 15,000) could be on the average 30 times more active in synthesis, as these sites provide the templates for induced (or unrepressed) enzymes. Such selected material could well be different in composition from the average DNA; thus, there is no reason to eliminate ribosome-like RNA as possible template material on the basis of its composition. In fact it is difficult to visualize how the DNA-like RNA of highly variable composition could act as templates for proteins of relatively constant composition.

The fraction of ribosome-like compositions is ultimately incorporated into ribosomes but its lifetime is sufficient to let it serve as template for 20–40 polypeptide strands.¹⁶ Thus, there is no kinetic evidence against its possible role as template.

At this time, it is not possible to choose with certainty among the alternatives presented, whether the code is triplet or mainly doublet, whether DNA-like or ribosome-like RNA, or both act as templates. As each alternative has advantages and failings, all deserve consideration until definitely eliminated.

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Comment. This paper was written for presentation at a symposium on coding held at the National Academy of Sciences. Its purpose (expressed in the closing paragraph) was to urge continued consideration of alternatives to the "dogma" until they were ruled out by experimental evidence.

At that time the hypothesis that newly formed ribosomal RNA provided the templates for a large proportion of the protein was completely tenable. Two months later, measurements by Yankofsky and Spiegelman (*Proc. Natl. Acad. Sci. U. S.*, **48**, 1069-1078 and 1466-1472, 1962) showed that ribosomal RNA was complementary to only a small portion of DNA. This finding was confirmed by Bolton and McCarthy (*J. Mol. Biol.*, in press). The portion of DNA involved (0.3-0.4 per cent) does not seem adequate to specify the variety of proteins found in bacteria.

The doublet proposal was even more heretical and was promptly denounced by Dr. S. Brenner as an "N.B.C. Code," meaning "Naive Biochemist's Code" or perhaps "Not Brenner's Code." The doublet is remarkably successful in predicting which amino acids will be incorporated in the presence of synthetic polymers. In fact, the doublet letters are (at present) necessary and sufficient for eleven of the amino acids. There are, however, failings which seem too frequent to attribute to the properties of the cell-free system. The successes are undoubtedly due to the fact that two of the bases in the code words are relatively more important. Richard B. Roberts.

III.C.13 Further Implications of the Doublet Code

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It is commonly assumed at present that the order of groups of nucleotides in RNA determine the order of amino acids in the protein which is assembled by using that RNA as a template. The "biological code" is the correlation of groups of nucleotides ("words") with amino acids. If more than one word corresponds to an amino acid, the words are said to be "degenerate"; if more than one amino acid corresponds to a word, the word is said to be "ambiguous." If no amino acid corresponds to a word, the word is designated "nonsense." If a group of three nucleotides designates an amino acid, the code is triplet and there are 64 possible words. If a group of two nucleotides designates an amino acid, the code is called a doublet and there are 16 possible words. It is evident that a doublet code must contain apparently ambiguous words since there are 20 amino acids which must be designated by 16 words.

At present the various experimental data which indicate the correspondence of amino acids to code words are usually interpreted in terms of a triplet code, but a number of inconsistencies appear in this interpretation. All of the words indicated by studies of the amino acid incorporation¹⁻⁷ stimulated by synthetic polynucleotides contain at least one uracil (U). If the templates for protein synthesis were

composed only of such words they would contain a higher proportion of U than is found in any natural RNA. If the triplet interpretation is to be retained, there must be other degenerate words of low U content which are more frequently used in natural template RNA.

Recent studies of the amino acid incorporation stimulated by synthetic polynucleotides of reduced U content⁷ have tested some 40 of the 64 possible triplet words. Of these, 20 seem to correspond to amino acids, three are possible degenerate words, and 17 seem to be nonsense. For example, a UC polynucleotide containing the 8 words which can be made from U and C was tested. Four of these accounted for the observed incorporation of four amino acids, so the other four must be judged nonsense. Other polymers gave similar results and no meaningful words lacking U were found. This high proportion of nonsense appears to be inconsistent with the interpretation of Crick *et al.*⁸ that the code is a triplet containing little nonsense.

In the same experiments,⁷ the UC polynucleotide which, according to the triplet interpretation, contains 60 per cent nonsense words, was very effective in stimulating amino acid incorporation into TCA precipitable polypeptides. If nonsense words act to terminate the formation of peptide strands, very few chains longer than five would be found.

The amino acid replacements which are caused by mutations give other clues to the correspondence of code words and amino acids. It is to be expected that most amino acid replacements are due to the change of a single base. Smith⁹ has shown that most of these replacements can be interpreted in terms of the presently discovered high-U triplet words if the U common to all of the words is assigned the same position in all of the words and all changes occur in the other two letters. Such a result seems unlikely if naturally occurring template RNA was mainly composed of a different set of low-U words.

These difficulties do not apply to the doublet code (Table 1). The same amino acid replacement data can be interpreted as single base changes in the words of the doublet (Table 2). It therefore seems appropriate to examine some of the further implications of the doublet code. From this point, it will be assumed that

TABLE 1
DOUBLET CODE

	G	A	C	U
G	gly try	glu gluN	ala	val
A	asp aspN	lys met	thr	ileu
C	arg	his	pro	leu
U	cys	tyr	ser	phe

Doublet code¹⁰ with aspN and met interchanged for greater symmetry.

the doublet code is correct and that the apparent ambiguities will ultimately be explained in terms of additional information carried by purine pairs in some specialized way. The doublet code is then to be used as a tool to determine some of the properties of template RNA. The justification for such a procedure comes from the numerical relationships which are uncovered.

1. The doublet code (Table 1) derived solely from amino acid incorporation and replacement data¹⁰ has the surprising feature that it groups the amino acids ac-

TABLE 2
AMINO ACID REPLACEMENT

Replacement	Interpretation	Replacement	Interpretation
glu → val	GA → GU	ser → thr	UC → AC
glu → lys	GA → AA	thr → aspN	AC → AG
glu → gly	GA → GG	val → phe	GU → UU
glu → gluN	GA → GA	thr → ser	AC → UC
val → glu	GU → GA	val → ileu	GU → AU
aspN → lys	AG → AA	phe → tyr	UU → UA
gly → asp	GG → AG	gly → glu	GG → GA
lys → asp	AA → AG	gly → arg	GG → CG
his → tyr	CA → UA	ileu → phe	AU → UU
his → arg	CA → CG	arg → leu	CG → CU
glu → ala	GA → GC		

Amino acid replacement data of Tables 2-4⁹ interpreted in terms of doublet code.

cording to their structure. If this grouping is meaningful and not simply due to chance, it implies that there may be direct and selective interactions between amino acids (or their activated forms such as adenylates) and the pairs of bases making up the code words of the template. This feature may be highly important as the doublet would be expected to provide only a weak interaction between an adapter S-RNA molecule and the template. The melting temperature of the complex between adenine dinucleotides and poly U is less than 10°, ¹¹ but cells can grow at 75°.

2. The frequencies of the amino acids of a protein can be entered into the matrix of the doublet code to indicate the frequency of the corresponding code words as shown in Table 3. The frequency with which each base occurs in one position of the pairs in the template is obtained by summing the columns; the frequency in the other position is obtained by summing the rows. In the first examples of Table 3,

TABLE 3

	G	A	C	U	Σ		G	A	C	U	Σ
	<i>E. coli</i>						<i>B. subtilis</i>				
G	8.6	11.2	10.6	7.2	37.6	G	9.3	11.8	9.1	7.6	37.8
A	10.4	9.1	5.6	5.4	30.5	A	10.2	9.9	5.9	5.0	31.0
C	5.0	2.1	4.2	8.9	20.2	C	4.2	2.2	4.2	8.6	19.2
U	0.6	2.8	4.7	3.5	11.6	U	0.0	2.9	5.1	3.9	11.9
Σ	24.6	25.2	25.1	25.0		Σ	23.7	26.8	24.3	25.1	
	Average protein						T2 bacteriophage				
G	9.7	10.7	8.7	6.8	35.9	G	10.4	11.8	7.6	5.9	35.7
A	9.7	8.7	5.8	4.8	29.0	A	11.6	8.5	5.9	6.5	32.5
C	4.9	1.9	4.8	8.7	20.3	C	5.0	0.9	3.9	5.9	15.7
U	1.9	2.9	5.8	3.9	14.5	U	0.4	6.3	5.3	5.5	17.5
Σ	26.2	24.2	25.1	24.2		Σ	27.4	26.5	22.7	23.8	
	Wool						Haemoglobin				
G	11.5	11.1	5.3	5.4	33.3	G	8.9	6.4	12.8	9.0	37.1
A	6.6	3.5	6.5	3.7	20.3	A	8.6	8.7	5.3	0.9	23.5
C	6.8	0.8	8.1	7.3	23.0	C	2.6	5.9	4.8	11.6	24.9
U	6.6	2.6	10.4	2.8	22.4	U	0.3	1.9	6.1	5.3	13.6
Σ	31.5	18.0	30.3	19.2		Σ	20.4	22.9	29.0	26.8	

Frequencies of amino acids in various proteins entered into corresponding positions of doublet code to give frequencies of code words in templates. Data for *E. coli* and *B. subtilis* proteins,¹² average proteins, most probable frequencies of amino acids in 34 analyses of 19 purified proteins and 15 different organs and organisms,¹³ T2,¹⁴ wool, and haemoglobin.¹⁵

where this procedure is applied to the average amino acid composition of a large number of proteins, each base occurs with approximately equal probabilities in one position. The distribution of bases in the other position (which we will call the second position) is nonuniform. The body of the table shows that the most fre-

quent pairs are purine-purine, G-C and amino-keto, indicating that familiar chemical forces influenced the probabilities of the pairs.

3. Individual proteins from specialized mammalian tissues (e.g., wool and haemoglobin in Table 3) do not show a uniform distribution of bases in the first position but they do show the same tendencies in the selection of the second member of the pair.

4. Table 4 shows the distribution of the pair frequencies for several RNA viruses. When these values are averaged (Table 5) it appears that the frequencies of the first position approximate those of the second if the bases are interchanged according to Watson-Crick pairing rules. Such a distribution would result if a template such

TABLE 4

	G	A	C	U	Σ		G	A	C	U	Σ
	Tobacco mosaic virus						Tomato bushy stunt virus				
G	4.9	10.4	9.2	8.5	33.0		9.1	5.7	8.5	10.0	33.3
A	11.6	1.2	10.4	5.5	28.7		11.1	4.2	11.0	3.3	29.5
C	6.7	0	4.8	7.9	19.4		5.3	1.2	3.9	10.9	21.3
U	0.6	2.4	11.0	4.8	18.8		0.8	2.8	8.6	3.6	15.2
Σ	23.8	14.0	35.4	26.7			26.3	13.9	32.0	27.8	
	Cucumber virus						Turnip yellow virus				
G	2.9	5.8	9.0	8.5	26.2		5.0	7.1	7.4	7.4	26.9
A	12.9	2.1	7.7	4.1	27.3		5.1	8.4	13.6	8.2	35.3
C	7.0	0	6.5	9.4	22.9		1.6	1.4	11.6	8.5	23.1
U	0	2.4	11.7	7.8	21.9		2.5	1.5	8.3	2.7	15.0
Σ	22.8	10.3	34.9	30.3			14.2	18.4	40.4	26.8	
	Polio virus						Southern bean mosaic virus				
G	6.8	7.7	7.8	7.2	29.5		9.0	6.8	7.7	6.8	30.3
A	11.9	6.2	9.1	4.8	32.0		7.3	5.6	12.4	6.0	31.3
C	4.7	2.4	7.2	8.5	22.8		6.4	1.3	4.7	8.5	20.9
U	0.8	3.9	7.0	4.4	16.1		0.9	4.3	9.0	3.4	17.6
Σ	24.2	20.2	31.1	24.9			23.6	18.0	33.8	24.7	

Frequencies of amino acids in virus proteins¹⁸ entered into corresponding positions of doublet code to give frequencies of code words in virus templates.

as the average template of *E. coli* duplicated by base pairing and subsequently both strands, the original and the complementary strand, served as template. The high proportion of G in the second position of the original strand would give rise to a high proportion of C in the first position of the replica. If each strand then served as template two proteins would be made. One would be normal with the usual high proportion of glycine, lysine, and glutamic acid, and the other would have a high content of proline, phenylalanine, and serine. The frequency of other amino acids such as alanine, arginine, tyrosine, and isoleucine would not be affected. A single

TABLE 5

		G	A	C	U
Average of 6 viruses	1st position	22.4	15.8	34.7	26.9
	2nd position	29.9	30.7	21.7	17.4
Expected from coli template and replica	1st position	22.6	18.3	31.3	27.8
	2nd position	31.3	27.8	22.6	18.3

Average frequency of first and second positions of virus templates compared with frequencies calculated for average of *E. coli* template and its replica.

protein, such as the virus coat, would not be expected to show evidence of contributions from two types of templates. If, however, the virus RNA mutated by joining its replica, from that time on, a single RNA strand could contain regions of both types and could contribute both distributions of amino acids to different

regions of the same protein. In any one virus, the two types would not be present in equal quantities. Thus, it is necessary to average a number of viruses before the underlying rules begin to emerge.

It is striking that the central region of the TMV protein¹⁷ has quite a different distribution of amino acids from the regions at the ends of the molecule (Table 6). An RNA having the proportions of bases which are found in templates for average proteins (e.g., *E. coli*) making two connections with its replica could give rise to this distribution of amino acids.

TABLE 6

Amino acids	Frequency in <i>E. coli</i>	Frequency from replica of <i>E. coli</i> template	Frequency in TMV No. 1-30 plus 137-157	Frequency in TMV 31-136
I. gly, glu, lys, met, val, thr	41.7	14.9	17.6	40.6
II. pro, ser, phe, his, cys	14.9	41.7	37.3	13.2
III. asp, leu, ala, arg, tyr, ileu	43.1	43.1	39.1	46.2

Frequency of amino acid groups in selected regions of TMV protein compared with frequency in *E. coli* and frequency expected from replica of *E. coli* template.

5. The simple rules for pair formation which can be deduced from the compositions of the templates provoke further speculation. It is obvious that such rules could not arise from the copying mechanisms which govern the biosynthesis of most nucleic acids. These relationships, if they are meaningful and not a coincidence, must provide an indication either of the stability of certain pairs or of the origin of the nucleic acid before the copying mechanism began to operate. Stability seems to be the less likely explanation. DNA shows, in its nearest-neighbor distribution,¹⁸ less evidence of these pairing rules than do the pairs calculated in the template.

At one time, nucleic acid must have polymerized *de novo* (i.e., without replicating a preexisting molecule). At that time, perhaps, the first member of each pair was drawn at random from equal supplies of the four nucleotides and the selection of the second member of the pair was influenced by the pairing rules. Such nucleic acids, if they served as templates, would produce proteins having roughly the same amino acid compositions as those of the present day. From that time on, the same pattern could be maintained by the copying mechanism in a small but significant part of the DNA

Alternatively, the process of nucleic acid formation *de novo* may have continued throughout evolution. Even if most of the nucleic acids were formed as copies of existing molecules, a very small part, formed according to simple pairing rules, could have a profound effect. In addition to the small changes resulting from point mutations, there would be the opportunity for organisms to produce completely new and different nucleic acids. Thus, new inventions would be possible as well as small modifications of existing designs.

As the numerical relationships discussed in this paper are the result of applying the doublet code to protein compositions, an underlying significance of the doublet formulations seems inescapable.

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Comment. The relationships noted in this paper remain for the most part unchanged, whether they are derived from a strict doublet code or from a triplet code in which the doublet portion is dominant. The grouping of amino acids in accordance with their structure may trace back to the time when life originated and specificity depended more directly on chemical forces. This point is discussed in detail by Woese (ICSU Reports, 1963). Richard B. Roberts.

III.C.14 The Kinetics of Transfer Ribonucleic Acid Synthesis in Escherichia coli

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SUMMARY

The kinetics of transfer RNA synthesis in *Escherichia coli* cultures has been studied, using [¹⁴C]uracil and [³²P]orthophosphate.

1. In exponentially growing cells, uracil is incorporated into transfer RNA, bypassing the intracellular nucleotide pool.

2. Both the uridine and pseudouridine nucleoside phosphate residues in transfer RNA are labeled by [¹⁴C]uracil at the same rate. The labeling of transfer RNA is delayed by about 1½ min, compared to the other RNA components.

3. If transfer RNA is labeled with [³²P]orthophosphate, the labeling of its uridine 5'-phosphate and pseudouridine 5'-phosphate residues suffers a greater kinetic delay than the uridine 5'-phosphate residues of the remainder of the newly formed RNA. At the same time, the entry of ³²P into all the RNA fractions is delayed by its flow through a large intracellular pool.

4. The kinetic delay of the entry of [¹⁴C]uracil into the uridine phosphate and the pseudouridine phosphate residues of transfer RNA is greatly lessened during the incubation of cultures in chloramphenicol.

5. If the newly formed RNA synthesized during incubation with chloramphenicol is incubated with homologous DNA-agar, it is found that hybridizable RNA is present in relatively greater quantity than in untreated control cultures.

6. From these findings, it is suggested that transfer RNA is synthesized from a "private" pool of material fed partly by the breakdown products of DNA-like RNA, and to a much lesser extent from the large intracellular pool of nucleotides implicated as a precursor for nucleic acid phosphate. The interconversion of pseudo-uridylic and uridylic acids incorporated into transfer RNA must occur in this "private" pool. By analogy, the identical kinetics of the labeling of transfer RNA and DNA by [¹⁴C]uracil and [³²P]orthophosphate suggests that the interconversion of ribonucleotides to deoxyribonucleotides for DNA synthesis also occurs in the same pool.

INTRODUCTION

Of the various RNA fractions known to exist in bacterial cells, about 20 % is in the form of soluble, or transfer, RNA (s-RNA). This RNA is held to be an intermediate

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Abbreviations: R-RNA, an RNA with the base composition of bacterial ribosomal RNA; D-RNA, an RNA with the base composition of the bacterial DNA.

in the synthesis of proteins from amino acids, through the activation and transfer of the latter to the ribosomes, the site of assembly of polypeptide chains¹.

Although much work has been done *in vitro* on the function and the metabolism of specific parts of the transfer RNA molecule, especially that more directly involved in the chemistry of amino acid activation², little has been done to determine the properties and synthesis of transfer RNA *in vivo*³.

Transfer RNA is unique in that it possesses several nucleotides which are probably not present in other RNA fractions⁴⁻⁶. The most abundant of these unusual nucleotides is pseudouridylic acid⁴ (5-ribosyluracil phosphate). Although relatively little is known about the biosynthesis of pseudouridylic acid and other minor nucleotides in transfer RNA, recent work has indicated that pseudouridylic acid may be synthesized from some form of uridylic acid in *Neurospora crassa*⁷. Other minor components, for example the methylated nucleotides, may be formed after completion of the polynucleotide chain of transfer RNA⁸.

An earlier paper⁹ described the kinetics of synthesis of transfer RNA and DNA in growing bacterial cells. It was suggested that both of these nucleic acids are synthesized from material in a small "private" pool fed partly by the breakdown products of D-RNA¹⁰ as well as from the large intracellular nucleotide pool. From these considerations, the kinetics of labeling of the uridylic and the pseudouridylic acid moieties of transfer RNA by either [¹⁴C]uracil or [³²P]orthophosphate were studied. This allowed the biosynthesis of pseudouridylic acid to be fitted into the general kinetics of nucleic acid synthesis, and provided stronger evidence for the existence of the "private" pool^{9,10}.

METHODS

The bacterium *Escherichia coli* ML 30 was the same strain used in all previous work⁹. Cultures growing exponentially at 37° were grown in aerated glucose-Tris media containing 8 mg/l phosphorus. The mean generation time of the cultures in these conditions was 50-55 min.

The preparation of de-proteinized RNA and the techniques of isolating transfer RNA by the use of a methylated serum albumin coated kieselguhr column¹¹ have been described⁹.

RNA was enzymically digested to nucleoside 5'-phosphates by snake venom phosphodiesterase (Worthington Biochemical Corporation). 100 µg of the enzyme was added to 5 ml of 0.02 M ammonium bicarbonate-0.01 M magnesium chloride buffer¹² (pH 8.6) containing 0.1-0.2 mg labeled RNA, and the mixture was incubated at 37° for 15 min to complete digestion. The specificity of the enzyme preparation was tested by comparing nucleotides liberated from ³²P-labeled RNA with commercial nucleoside 5'-monophosphates (California Corporation for Biochemical Research), by Dowex-1 formate ion-exchange chromatography.

The method of alkaline digestion of RNA has been previously described¹³.

Separation of pseudouridylic and uridylic acids from either alkaline or enzymic digestion of RNA was carried out by ion-exchange chromatography on Dowex-1 X8 formate columns (200-400 mesh), using a non-linear gradient of formic-acidammonium formate¹⁴.

1. The separation of the nucleoside 5'-phosphates resulting from enzyme digestion of RNA was carried out as follows. After loading the hydrolyzate onto the column,

200 ml of 0.15 M formic acid was passed to elute the bulk of the cytidine and adenosine 5'-phosphates. 30 ml of 0.01 M formic acid was then passed. A nonlinear gradient of formic acid-ammonium formate was begun, using five serially connected vessels each of 80 ml capacity. These contained the solutions listed in Table I. This gradient was sufficient to elute and resolve pseudouridine 5'-phosphate and uridine 5'-phosphate (Fig. 1). The fractions containing pseudouridine 5'-phosphate were then pooled and made alkaline with 0.3 M ammonium hydroxide. This solution was loaded onto a

TABLE I
NONLINEAR GRADIENT OF FORMIC ACID-AMMONIUM FORMATE FOR ELUTION OF NUCLEOSIDE 5'-PHOSPHATES

Vessel No.	Ammonium formate (M)	Formic acid (M)
1	0.015	0.01
2	0.03	0.01
3	0.05	0.01
4	0.06	0.01
5	0.08	0.05

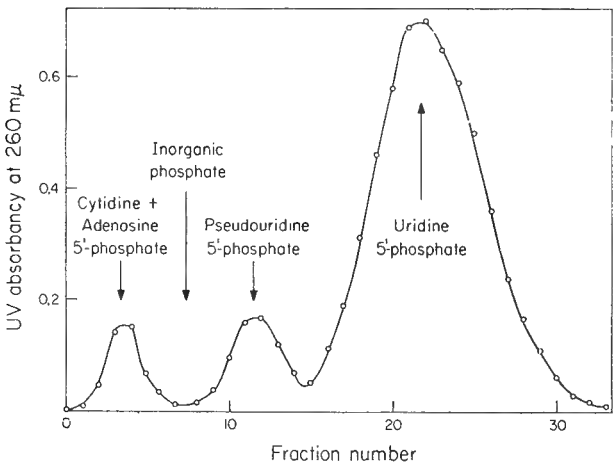


Fig. 1. Dowex-1 formate ion-exchange chromatography of nucleoside 5'-phosphates from phosphodiesterase digests of transfer RNA.

Dowex-1 column equilibrated with ammonium hydroxide, and the column was then converted to the carbonate form by washing through 100 ml of 0.1 M sodium carbonate. This effectively removed any remaining cytidine and adenosine 5'-phosphates. 100 ml of 0.4 M ammonium bicarbonate buffer (pH 8.6) was then passed, eluting the pseudouridine 5'-phosphate. The pooled fractions were then concentrated at 60° under reduced pressure to 3 ml, degrading the ammonium bicarbonate. The remaining solution was made up to 0.05 M formic acid-0.05 M ammonium formate to prepare for counting.

2. Alkaline digests of transfer RNA were treated in a similar way. In this case,

the cytidine and adenosine 2',3'-phosphates were first eluted with 200 ml of 0.2 M formic acid. A five-chamber gradient was then set up, containing the solutions listed in Table II. This gave complete resolution of pseudouridine 2',3'-phosphates and uridine 2',3'-phosphates (Fig. 2). If necessary, the pseudouridine 2',3'-phosphates were concentrated by the above procedure.

TABLE II
NONLINEAR GRADIENT OF FORMIC ACID-AMMONIUM FORMATE FOR ELUTION OF NUCLEOSIDE 2',3'-PHOSPHATES

<i>Vessel No.</i>	<i>Ammonium formate (M)</i>	<i>Formic acid (M)</i>
1	0.02	0.01
2	0.04	0.01
3	0.05	0.01
4	0.05	0.05
5	0.10	0.10

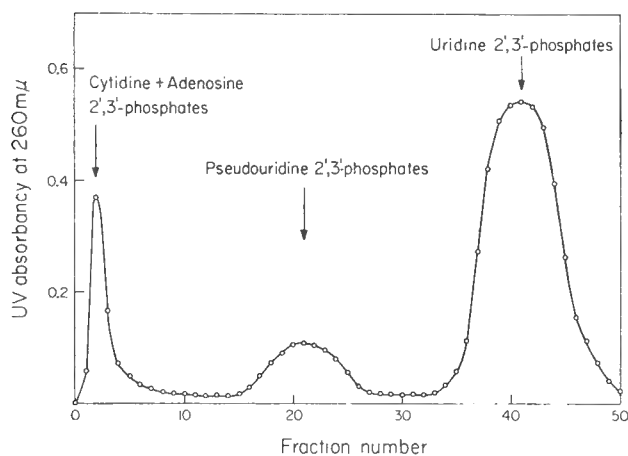


Fig. 2. Dowex-1 formate ion-exchange chromatography of nucleoside 2',3'-phosphates from alkali digests of transfer RNA.

The positioning of the pseudouridine and uridine phosphates on the chromatograms was determined by the spectrophotometric analysis of nucleotides purified from hydrolyzates of yeast nucleic acid.

The methods of labeling with [^{32}P]orthophosphate and [^{14}C]uracil and the simultaneous counting of the isotopes have been previously described¹⁵. For the accurate counting of doubly labeled nucleotides in the Packard Tri-Carb Liquid Scintillation Counter (Packard Instrument Co.) a water-miscible counting medium was used¹⁶. When 3 ml of the sample, containing formic acid-ammonium formate in the concentration range normally encountered in the chromatography was added, minimization of ^{32}P contamination in the 0-50 V channel, combined with a reasonable efficiency of ^{14}C -counting, was obtained at a setting of 1080 V.

Nucleotide base compositions were measured as previously described¹³.

D-RNA was separated from phenol-treated RNA preparations by the DNA-

agar column method¹⁷. For this particular experiment *E. coli* BB was used to prepare both the labeled RNA and the DNA of the column. Elution of more than 80 % of the hybridized RNA from the column was effected by passing 70 % methanol at 60°.

Chloramphenicol (Parke Davis Co.) was used at a concentration of 200 mg/l. [$2\text{-}^{14}\text{C}$]Uracil was a product of New England Nuclear Corporation.

RESULTS

The pseudouridylic acid content of rapidly labeled RNA

Previous work⁹ suggested that the turnover of D-RNA in growing bacteria partly supplies the pool from which material for DNA and transfer RNA synthesis is drawn. An estimate of the content of pseudouridylic acid in the r4-S rapidly labeled RNA fraction (D-RNA + R-RNA) is important to determine the extent of any pseudouridylic-uridylic acid interconversions.

An exponentially growing culture of *E. coli* ML 30 was labeled for 2 min with [^{32}P]orthophosphate. At this time, virtually all the labeled RNA is in the r4-S fraction^{13,15,18}. After phenol extraction of the broken cells⁹, the total RNA of the pulse-labeled sample was precipitated by cold 5 % trichloroacetic acid, and filtered. The whole filter was then hydrolyzed by alkali¹³ and the labeled nucleotides were separated as described earlier, to compare pseudouridine and uridine 2', 3'-phosphate contents of the labeled RNA. Fig. 3 shows that there is a negligible amount of pseudouridylic acid present in newly formed RNA. Thus it is unlikely that the pseudouridine phosphate content of transfer RNA is derived directly from pseudouridylic acid moieties present in r4-S RNA (D-RNA + R-RNA).

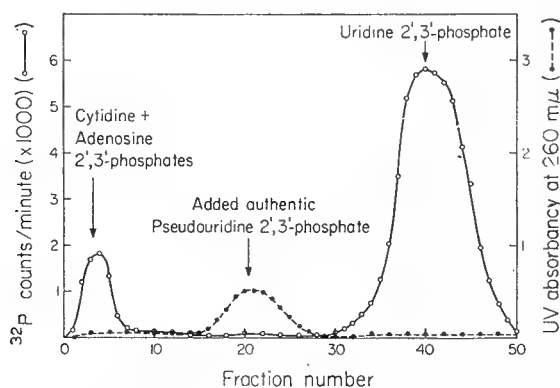


Fig. 3. Dowex-1 formate ion-exchange chromatography of ^{32}P -labeled nucleoside 2',3'-phosphates from alkali digests of r4-S rapidly labeled RNA. The RNA was labeled by 2 min exposure of a growing culture to [^{32}P]orthophosphate. ●-●, ultraviolet absorbancy at 260 mμ of added unlabeled pseudouridine 2',3'-phosphate; O-O, ^{32}P counts/min.

The pseudouridylic acid content of the intracellular nucleotide pool

An attempt was made to estimate the size of the pseudouridylic acid pool in growing cultures, by labeling the cells with [^{14}C]uracil. A growing culture of *E. coli* ML 30 at a concentration of 1 g/l was labeled with [^{14}C]uracil for 5 min. After chilling and centrifugation, the cells were suspended in cold 75 % ethanol and again centrifuged. The supernatant contained the intracellular nucleotide pool of nucleoside

5'-mono- di- and triphosphates. This mixture was then treated with an equal volume of 0.2 M HCl and was heated in a boiling water bath for 10 min to convert the bulk of the pyrimidine nucleoside 5'-polyphosphates to monophosphates. After the removal of most of the HCl by evaporation under reduced pressure, chromatography on Dowex-1 formate columns was carried out to separate the nucleotides. No radioactivity could be detected in the position of the chromatogram normally occupied by pseudouridine 5'-phosphate. It must be concluded that the pool content of pseudouridylic acid is less than one percent of the uridylic acid. There is, thus, no large pool of pseudouridylic acid present which might cause an appreciable delay in the labeling of pseudouridylic acid residues of transfer RNA.

Kinetics of labeling of transfer RNA, D-DNA and R-RNA

The kinetics of labeling of bacterial RNA's by [^{14}C]uracil or by [^{32}P]orthophosphate were observed as described earlier⁹. The results are identical with those recorded in this earlier paper. The entry of [^{14}C]uracil into transfer RNA and into DNA was delayed by about 1-1.5 min, and the entry of [^{32}P]orthophosphate into both transfer RNA and DNA was similarly delayed relative to the remainder of the RNA. The paper referred to above contains the results relevant to such experiments.

Kinetics of labeling of transfer RNA pseudouridine 5'-phosphate and uridine 5'-phosphate moieties

Labeling with $^{32}\text{PO}_4^{3-}$: An exponentially growing culture randomly labeled with [^{14}C]uracil for more than two generations was labeled with [^{32}P]orthophosphate. At suitable intervals, samples of the culture were removed, poured onto crushed frozen medium, centrifuged and broken in the French pressure cell in the presence of 0.2 % sodium dodecyl sulfate. The effluent was immediately treated with phenol, and the RNA purified by chromatography on the methylated serum albumin coated kieselguhr column⁹. The various RNA fractions together with the NaCl used to elute them were pooled and concentrated to 2 ml. The solutions were then passed through a Sephadex G-25 column, equilibrated with 0.02 M ammonium bicarbonate-0.01 M MgCl_2 buffer (pH 8.6) to remove the salt by gel filtration. Snake venom phosphodiesterase was added to the RNA fractions and the RNA hydrolyzate was chromatographed on Dowex-1 formate columns. After concentration of the pseudouridine 5'-phosphate, both it and uridine 5'-phosphate were counted. The change in specific activities of the transfer RNA pseudouridine 5'-phosphate and uridine 5'-phosphate, and of the uridine 5'-phosphate of the remainder of the RNA is indicated in Fig. 4. Since the nucleotide moieties were incorporated into the various RNA's as nucleoside 5'-phosphates, hydrolysis by snake venom phosphodiesterase breaks down the RNA into nucleotides possessing the same phosphate group incorporated with them originally. This experiment therefore allows a valid comparison between the ^{32}P -labeling of pseudouridylic and uridylic acids in the RNA.

Labeling with [^{14}C]uracil: This experiment was essentially the inverse of the previous one, the culture being randomly labeled with [^{32}P]orthophosphate and then labeled with [^{14}C]uracil. After separation of transfer RNA and ribosomal RNA in each sample, alkaline hydrolysis of the RNA's was carried out to give a mixture of nucleoside 2'- and 3'-phosphates.

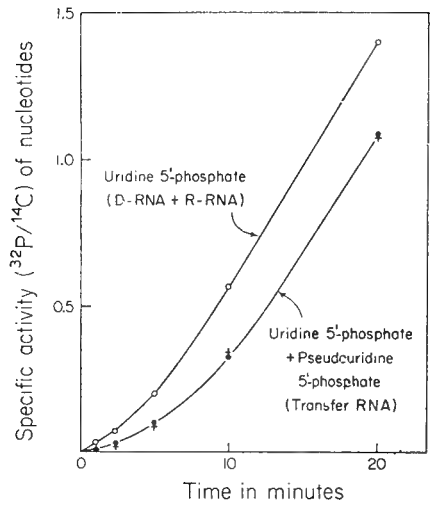


Fig. 4. Specific radioactivities of transfer RNA pseudouridine and uridine 5'-phosphate residues (●-●, +--+) and the uridine 5'-phosphate of D-RNA + R-RNA (○-○) as a function of ^{32}P incorporation time plotted as a ratio of ^{32}P counts/min to ^{14}C counts/min. Data from phosphodiesterase digests of purified transfer RNA and D-RNA + R-RNA.

Fig. 5 shows the changes in specific activity of transfer RNA pseudouridine and uridine 5'-phosphates and (D-RNA + R-RNA) uridine 5'-phosphate. Transfer RNA nucleotides were labeled with a 1-1.5-min kinetic delay, compared to those of the remainder of the RNA. The kinetics of incorporation of [^{14}C]uracil into the D-RNA + R-RNA indicates the intracellular pool of nucleotides is bypassed by the added

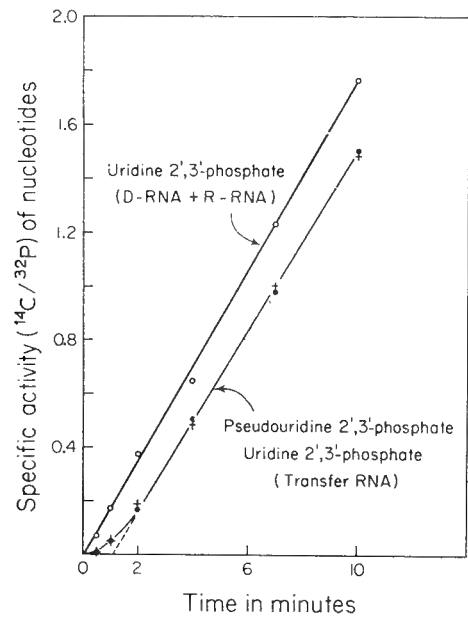


Fig. 5. Specific radioactivities of transfer RNA pseudouridine and uridine 2', 3'-phosphate (○-○, +--+) and D-RNA + R-RNA uridine 2',3'-phosphates (○-○) as ratios of ^{14}C counts/min to ^{32}P counts/min plotted against time of [^{14}C]uracil incorporation.

base^{19,20}. Also notable is the equal specific activity of transfer RNA pseudouridine 2',3'-phosphates and uridine 2',3'-phosphates at all times during [¹⁴C]uracil incorporation.

Labeling with [¹⁴C]uracil in the presence of chloramphenicol: A similar experiment was carried out in the presence of 200 mg/l chloramphenicol⁹. The pseudouridine and uridine 2',3'-phosphates of transfer RNA were purified as described in the previous section. Fig. 6 shows the changes in specific activity of the various uridylic acid fractions during the incorporation of [¹⁴C]uracil.

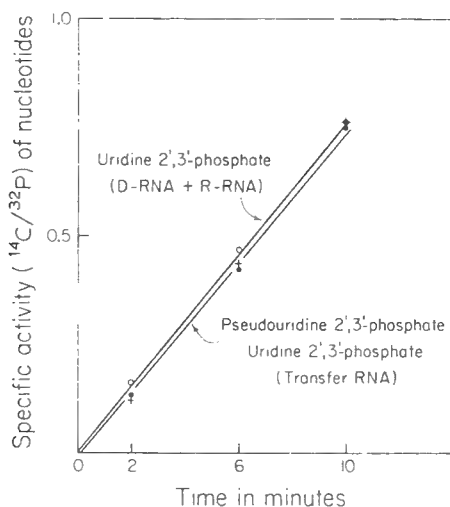


Fig. 6. Specific radioactivities of transfer RNA pseudouridine and uridine 2',3'-phosphates (●-●, +-+) and D-RNA+R-RNA uridine 2',3'-phosphates (O-O) as a function of time, during [¹⁴C]uracil incorporation into chloramphenicol-inhibited cultures.

The specific activities of the uridylic acids from transfer RNA are now almost identical with the uridylic acids from the remainder of the RNA. The kinetic delay of labeling of transfer RNA uridine nucleotides is greatly diminished in the presence of chloramphenicol and cannot be greater than about 20 sec (see ref. 9).

The effects of chloramphenicol incubation on D-RNA synthesis and turnover

An exponentially growing culture of *E. coli* BB at a cell concentration of about 1 g/l was divided into two parts. To one part, chloramphenicol was added to a concentration of 200 mg/l. Growth of the cells immediately stopped, although RNA synthesis continued. After 5 min incubation, both the chloramphenicol treated and the control cultures were pulse-labeled with 2 mC ³²PO₄³⁻. At labeling times of 2 min, 10 min and 30 min, samples of the culture were chilled, centrifuged and broken into a phenol-0.2 % sodium dodecyl sulfate mixture. After heating to 60° and mixing for 5 min, the aqueous phase containing RNA was cooled, extracted 5 times with ether and passed twice through a Dowex-50 ion-exchange column, equilibrated with 0.015 M sodium citrate-0.15 M sodium chloride buffer (pH 6.8). This treatment effectively removed ribonuclease activity. The RNA was then hybridized on *E. coli* BB DNA-agar as described elsewhere¹⁷.

Table III shows the percentage of hybridized labeled RNA present in each of the control and chloramphenicol-treated cultures. The nucleotide base composition of hybridized and unhybridized RNA from the 10-min pulse-labeled sample of chloramphenicol-treated cells is shown in Table IV. The hybridized RNA is essentially like the DNA in composition (uracil substituting for thymine) whilst the nonhybridized RNA is ribosomal in composition¹⁷.

TABLE III

HYBRIDIZATION OF RNA FORMED DURING CHLORAMPHENICOL INCUBATION, WITH HOMOLOGOUS DNA-AGAR

Time of labeling with ³² P (min)	Chloramphenicol-incubated culture		Control	
	Nonhybridized labeled RNA (%)	Hybridized labeled RNA (%)	Nonhybridized labeled RNA (%)	Hybridized labeled RNA (%)
2	67.2	32.8	69.9	30.1
10	83.9	16.1	91.3	8.7
30	95.0	5.0	96.5	3.5

TABLE IV

BASE COMPOSITION OF HYBRIDIZED AND NONHYBRIDIZED RNA IN TABLE III
10 min ³²P-labeled "Chloramphenicol" RNA

Nucleotide	Nonhybridized RNA (mole %)	Hybridized RNA (mole %)
C	22.1	25.6
A	24.8	24.0
G	31.1	26.4
U	22.0	24.0

The diminished breakdown of D-RNA in chloramphenicol-incubated cultures is strongly indicated by the results of Table III. An accurate estimation of the slower rate of breakdown of D-RNA is difficult, due to the combination of a possible differential acceleration of some RNA synthesis²¹. However the results indicate that the rate of D-RNA breakdown in chloramphenicol-treated cultures may be as little as one half the rate in normally growing cells.

DISCUSSION

The kinetics of nucleic acid biosynthesis in growing *E. coli* cultures has been described in detail^{9,15,19,22,23}. From these results all the known facts have been reconciled in a diagram¹⁰ showing the flows of exogenous and endogenous nucleotide bases and exogenous phosphate in the synthesis of the several nucleic acid fractions in the cells.

By following the incorporation of exogenously added bases into the nucleic acids of bacteria, it has been shown that the large intracellular pool of mononucleotides is not an obligatory precursor in RNA synthesis^{19,20}. BRITTEN¹⁰ studying the endogenous incorporation of [¹⁴C]formate into RNA purines, and MIDGLEY²⁴ studying the endogenous incorporation of [¹⁴C]aspartic acid into the RNA pyrimi-

dines, have found that the intracellular nucleotide pool is also bypassed by the flow of endogenously formed bases into nucleic acids. However it is obvious that there is a large pool existing as an intermediate in the incorporation of nucleic acid phosphate from exogenous inorganic phosphate. These findings explain the differences in the kinetic delays of RNA labeling demonstrated in the experiments.

It has been indicated that transfer RNA and DNA are synthesized from material drawn from a "private" pool fed in part by the products of D-RNA degradation⁹. The low initial rate of [^{14}C]uracil entry into transfer RNA requires the "private" pool to be fed by D-RNA breakdown as a major part of the in-flow of material. The present work, in extending the kinetic studies of transfer RNA synthesis, has shown that both the uridine and pseudouridine 5'-phosphate moieties in transfer RNA are derived from this "private" pool.

As no pseudouridylic acid was detected in alkali digests of rapidly labeled RNA (D-RNA + R-RNA), this finding, together with the identical kinetics of the labeling of transfer RNA uridylic and pseudouridylic acids by [^{32}P]orthophosphate and [^{14}C]uracil suggests that an interconversion of some form occurs in this pool. The formation of pseudouridylic acid endogenously probably occurs by synthetic pathways involving a uridylic acid derivative as an intermediate. Support for this hypothesis has come from the work of HALL AND ALLEN⁷ on pseudouridylic acid synthesis by a pyrimidine requiring mutant of *Neurospora crassa* and from POLLAK AND ARNSTEIN²⁵ by their studies on *E. coli*.

Since the kinetics of labeling of DNA are identical to those for the labeling of transfer RNA in these experiments⁹, by analogy it is suggested that the interconversion of ribonucleotides to deoxyribonucleotides also occurs in the "private" pool where pseudouridylic acid is formed. Therefore the possible reactions in the "private" pool from which material for transfer RNA and DNA synthesis is drawn are as shown in Fig. 7. There is also some further evidence supporting this general diagram, since

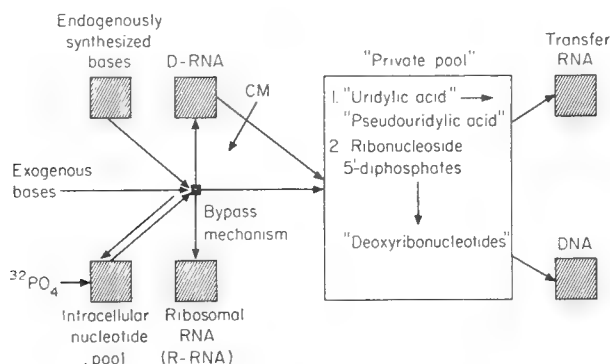


Fig. 7. Interrelationships between D-RNA, transfer RNA and DNA in *E. coli* suggested by data of this paper and others^{9,10}.

bacteriophage DNA may be synthesized by the partial utilisation of virus specific RNA degradation products in phage-infected cells²⁶. An autocatalytic degradation mechanism for *E. coli* ribosomal RNA has been demonstrated²⁷, whereby nucleoside 5' mono- and diphosphates are formed, presumably by the action of a diesterase and

polynucleotide phosphorylase, which has been found to be located predominately in the ribosomes^{28,29}. Since other work^{30,31} has indicated that nucleoside 5'-diphosphates are preferred precursors of deoxyribonucleotides, the outlined biochemical reactions shown in Fig. 7 may be roughly representative of the metabolism of the nucleic acids synthesized from the "private" pool.

The action of chloramphenicol, in lowering the rate of degradation of D-RNA, must affect the kinetics of transfer RNA and DNA synthesis from this pool by forcing an increased direct entry from the bypass mechanism past D-RNA as indicated by BRITTEN¹⁰, thus lessening the kinetic delay in the labeling of transfer RNA and DNA. It is possible that, in the presence of chloramphenicol, D-RNA is also broken down into degradation products which may be unsuitable for the synthesis of transfer RNA or DNA, for example the nucleoside 3'-phosphates formed by the action of ribonuclease. However, the principal factor in the lessening of the kinetic delay of transfer RNA labeling by [¹⁴C]uracil must be a slower rate of breakdown of D-RNA in chloramphenicol-incubated cultures.

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D. Related Material from Carnegie Year Books

III.D.1 Sedimentation Constants of Ribosomes

(Reprinted from Carnegie Institution of Washington Year Book 58, pp. 266-269, 1959.)

R. J. Britten and B. J. McCarthy

Sedimentation Constants of Ribosomes

In the previous annual report (Year Book 57) the four major sizes of ribosomes were described (sedimentation constants approximately 20, 40, 60, and 80S) and also the magnesium requirement for their stability. During the past year nearly 400 runs have been made with the analytical ultracentrifuge both to monitor preparations for different types of experiments and for further study of the nature and stability of the ribosomes.

The previously quoted apparent sedimentation constants were observed in total cell juices. Correction for the viscosity of the cell juices and studies of purified ribosome preparations showed that there are ribosomes of sedimentation constants of about 20, 30, 50, 70S and a larger component which varied in sedimentation constant from just over 70 to about 100S depending on the magnesium concentration and the growth condition of the bacteria. These sedimentation constants have been rounded off for convenience in discussion. Exhaustive studies of sedimentation as a function of concentration have not been made. The concentration dependence is small, however, and these figures are accurate to ± 5 per cent and consistent with published values for purified ribosomes.

Figure 14, plate 2, shows the Schlieren diagram of a total juice from exponentially growing cells. In this experiment the cells

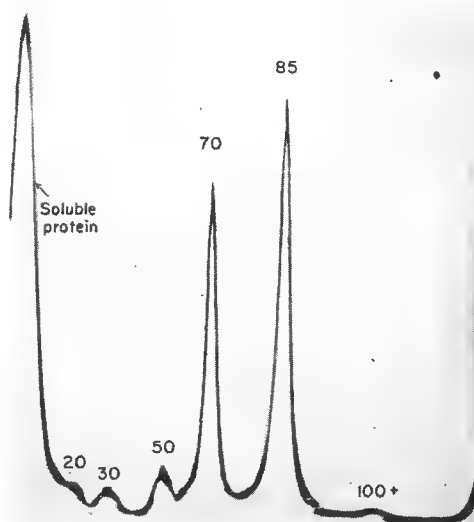


Fig. 14. Ribosomal pattern of cells of *E. coli* exponentially growing in broth. Cells washed and broken in TS at 10^{-2} M Mg. Schlieren plate taken 5 minutes after the analytical ultracentrifuge had reached 56,740 rpm. The peaks are labeled with nominal sedimentation constants. In comparison, cells grown in a salts-glucose medium show relatively less of the 70 and "85" S peaks.

were washed three times in TSM (10^{-2} M Mg). This magnesium concentration is high enough to drive the equilibrium among the various forms of ribosomes over

have very different specific radioactivities from the larger forms. Thus, these small forms are not simply dissociation fragments from the larger ribosomes and are

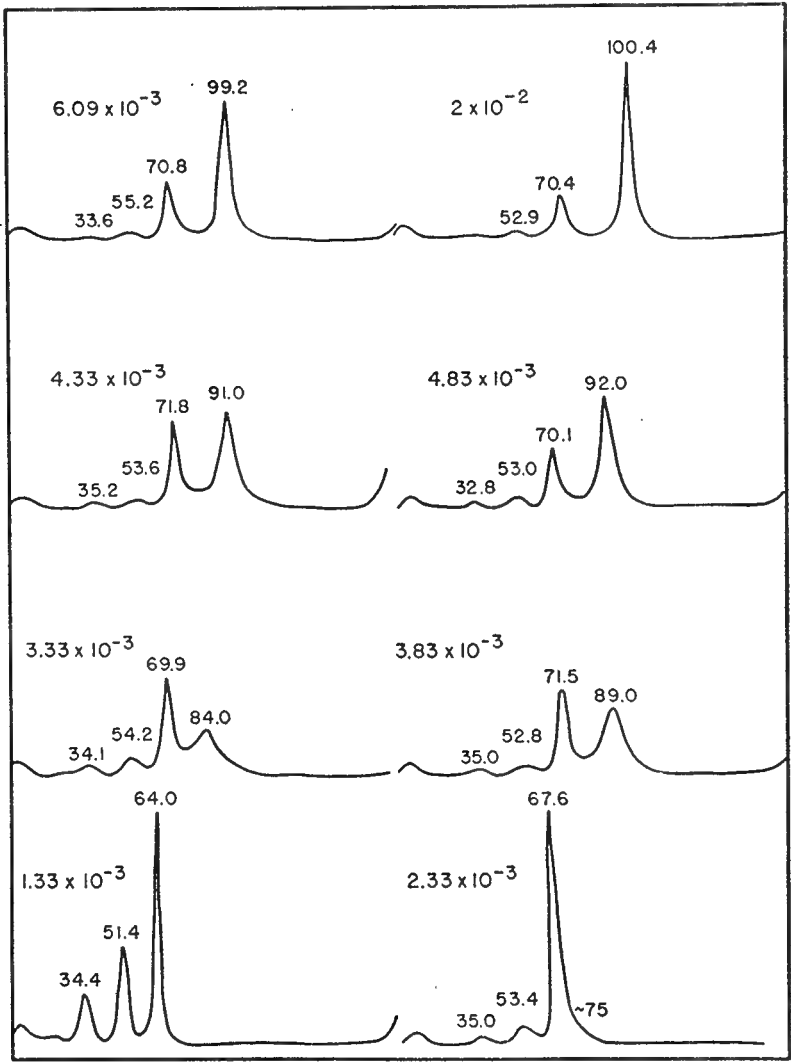


Fig. 15. Ribosomal pattern as a function of magnesium concentration. Tracings of 10× enlarged prints of Schlieren plates taken 5 minutes after the analytical ultracentrifuge had reached 50,740 rpm. The numbers at the left are the calculated magnesium concentrations in moles per liter. The numbers over the peaks are the measured sedimentation constants, corrected only for the viscosity change of water to 20° C. Ribosome preparation described in text.

toward the larger (70 and 100S) forms, but sizable quantities of small ribosomes (20, 30, 50S) still remain. A number of tracer experiments have shown that these smaller, naturally occurring particles may

identified for purposes of discussion as the small “native” ribosomes.

Figures 15, 16, and 17 show the results of a study of the effects of magnesium concentration on the quantity and sedi-

mentation constants of the various forms of ribosomes. Exponentially growing (broth) cells were washed three times in TS containing $2 \times 10^{-2} M$ MgAc, broken in the pressure cell, and centrifuged to

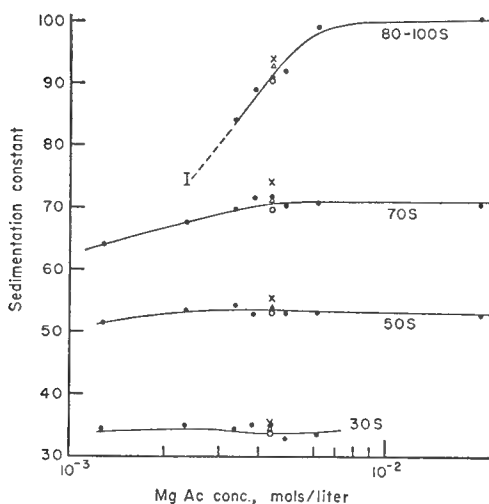


Fig. 16. Variation of sedimentation constant of the various forms of ribosomes with magnesium concentration. Data from figure 15.

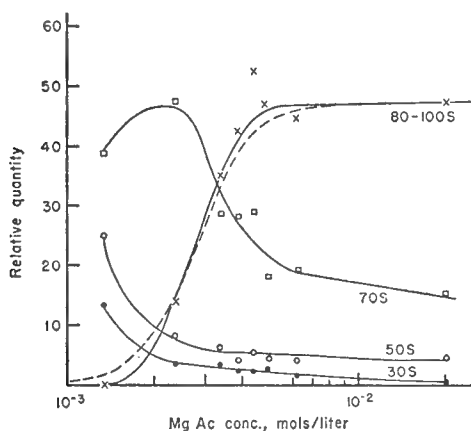


Fig. 17. Variation in the quantity of the various forms of ribosomes as a function of magnesium concentration. Data obtained from the Schlieren diagrams of figure 15 simply by cutting out and weighing the peaks from enlarged prints.

remove cell bodies and wall and membrane fragments. Then the larger ribosomes were harvested by centrifuging 45 minutes at 105,000g. These were then resuspended in a small volume of the

same buffer (TS $2 \times 10^{-2} M$ Mg). Small samples of this concentrated ribosome suspension were diluted a factor of 14 (to 3 mg RNA per ml) into TS to give the magnesium concentrations indicated. These calculated concentrations are not corrected for the magnesium due to dissociation of the Mg-ribosome complex.

The major observations of the effect of magnesium on the ribosomes may be summarized as follows: (1) Decreasing the magnesium concentration from 6×10^{-3} to $2 \times 10^{-3} M$ converts the 80 to 100S form into the 70S form without the appearance of any significant quantity of 50 and 30S forms. (2) At concentrations below $2 \times 10^{-3} M$ Mg the 70S ribosomes dissociate into 50 and 30S forms. (3) Removal of all magnesium (and other polyvalent cations) with EDT dissociates the 50 and 30S ribosomes in turn, with the consequent digestion of their RNA by the RNAase contained in the ribosomes. (4) The sedimentation constant of the 70S form is slightly dependent on the magnesium concentration below $3 \times 10^{-3} M$ Mg. (5) The sedimentation constant of the 80 to 100S form is strongly dependent on the magnesium concentration between 2 and $6 \times 10^{-3} M$ Mg. (6) The peak of variable sedimentation constant is noticeably broader at the intermediate magnesium concentrations. (7) The 70S and 80 to 100S forms exist in equilibrium with each other since reduction of the magnesium concentration from $2 \times 10^{-2} M$ to $1.8 \times 10^{-3} M$ and restoration to $4.33 \times 10^{-3} M$ yields the same picture (after overnight storage at $4^\circ C$) as was obtained by direct dilution of the magnesium concentration to $4.33 \times 10^{-3} M$. (8) Dilution of the ribosome concentration (to 1 mg RNA/ml) does not change the relative quantity of the 70 and 90S peaks at $4.33 \times 10^{-3} M$ Mg either immediately or after storage overnight at $4^\circ C$.

It appears that the 70S particle is a combination of 30S and 50S forms in equal numbers. If the 30, 50, and 70S particles were each approximately spherical and of

the same hydration and density, one 30 and one 50 might form a 70S particle. On the other hand, if the 70S form were a relatively more extended object it is possible that it could consist of two 30S and two 50S particles. The choice between these possibilities cannot yet be made with certainty.

Again, if the 70S were spherical and two 70S could dimerize to form a sphere of similar hydration and density the resultant particle would have a sedimentation constant of about 110S. Standing against this, however, are items 5 and 8 in the list above. The smooth change of sedimentation constant with magnesium concentration suggests an object of magnesium-dependent shape or hydration. The shape change required is quite striking since the transformation of a sphere to an ellipsoid of axial ratio 6 of identical mass and hydration would be necessary to reduce the sedimentation constant by about 30 per cent.

An alternative interpretation is that the intermediate values of sedimentation constant measure the average sedimentation rate of objects that are rapidly changing from a 70S to a 100S form and indicate the fraction of time the particle spends in each of the states. The presence of a 70S peak as the magnesium concentration is lowered through the critical range requires an additional hypothesis that the 70S particle exists as well in a state incapable of making the transformation. If a *rapid* transformation were due to a dimerization reaction one would expect that the equilibrium would be shifted in favor of the 70S form by reduction of the ribosome concentration. Since this is not observed it appears that variations of shape or hydration have a major effect on the sedimentation constants of the ribosomes and

may even totally account for the transition from the 100S to the 70S form. Attempts to settle this question by means of light-scattering measurements of the molecular weights have not yet been successful, owing to the presence of small quantities of wall or membrane fragments of enormous molecular weight but of sedimentation constants in the 70 to 100S range.

On figure 17 is shown a theoretical curve (dotted) representing the quantity of the 80 to 100S form expected for a transformation which depends upon the fifth power of the magnesium concentration. The implication of the good fit with the experimental data is that roughly 5 Mg atoms are required to trigger the transformation. Other published data indicate that there may be 1000 Mg atoms total absorbed per ribosome. If the shape change were a result of the formation of magnesium bonds which caused cross linking and contraction of an initially fairly open structure it is reasonable to suppose that a few magnesium atoms in a critical region could initiate such a transformation by drawing the strands together and thus allowing other cross-linking bonds to be formed sequentially down the length of the strands.

The relative quantity of 70 and 80 to 100S particles depends on the growth condition of the cells. The quantity of nascent protein associated with the 70 and 80 to 100S particles is observed to be different. These results imply that the transformation between these two forms is intimately associated with the growth of the cell and the synthesis of protein. A shape change of the ribosomes might be useful in freeing newly synthesized protein from the intimate association with the ribosomes that must exist during synthesis.

Comment. Further evidence about the nature of the 80S-100S peak is now available. Measurements carried out by K. E. Van Holde during a visit to the Department of Terrestrial Magnetism appear to support the idea that the 100S ribosome is a dimer of the 70S ribosome. These two species appear to be in equilibrium; measurements of the fraction of the material in the fast peak, corrected for the Johnson-Ogston effect, show it to be a function of total ribosome

concentration at $[Mg^{++}] = 3.75 \times 10^{-3} M$. The data are consistent with a simple dimerization mechanism, if it is to be postulated that 10-15 per cent of the 70S particles are incapable of dimerization.

The variation of the sedimentation coefficient of the fast peak with $[Mg^{++}]$ prompted a theoretical reexamination of the sedimentation of chemically reacting systems. It was shown (K. E. Van Holde, *J. Chem. Phys.*, **37**, 1922, 1962; Geneva Belford and R. L. Belford, *ibid.*, **37**, 1926, 1962) that, for reversible chemical reactions which are rapid but not infinitely fast, the sedimentation pattern observed should resemble those found for the 70S-100S ribosome system. In particular, the existence of a broad peak of intermediate sedimentation rate, the position of which should depend upon the position of the equilibrium, was predicted. K. E. Van Holde and Roy J. Britten.

III.D.2 Ribonuclease--Self-Digestion RNase

(Reprinted from Carnegie Institution of Washington Year Book **58**, pp. 276-278, 1959.)

E. T. Bolton

Ribonuclease. This activity is demonstrable when ribosomes are dissociated by treatment with 4.5 M urea or the chelator ethylenediaminetetraacetic acid. Upon treatment of ribosomes with either of these agents a rapid release of acid-soluble ultra-violet-absorbing components occurs. Most of the enzymic activity, as indicated by the digestion of added RNA, is found in the ribosomes of a cell juice, figure 25. The small amount (~10 per cent) found in the supernatant fluid after the ribosomes had been separated out is also latent. Since the supernates are rarely free of ribosomes it may be inferred that all the enzyme is particle-associated. After 10 minutes at 37° C in 4.5 M urea essentially all the RNA of ribosomes is degraded, figure 26. The time for complete degradation is the same for mixtures of ribosomes, or purified 70S, 50S, and 30S particles, and is the same whether the particles have been derived from cells growing in broth or in

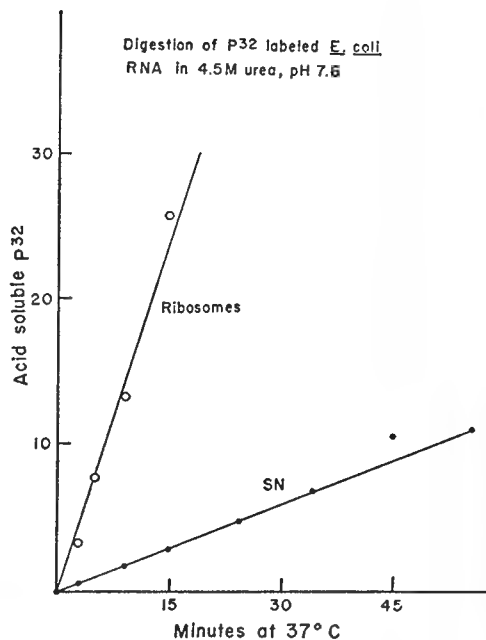


Fig. 25. Ribonuclease activity of ribosomes and the soluble (SN) fraction of *E. coli*. The amount of enzyme is indicated by the slope of each line.

synthetic media, or from cells which were nongrowing as a result of having exhausted the glucose supply. Thus, a constant amount of enzyme activity in relation to the total amount of nucleic acid in the ribosomes is observed. Since a given enzyme activity implies the existence of unique molecules that have the capacity to act specifically, it may be inferred that there is a constant proportion of ribonuclease molecules in ribosomal particles.

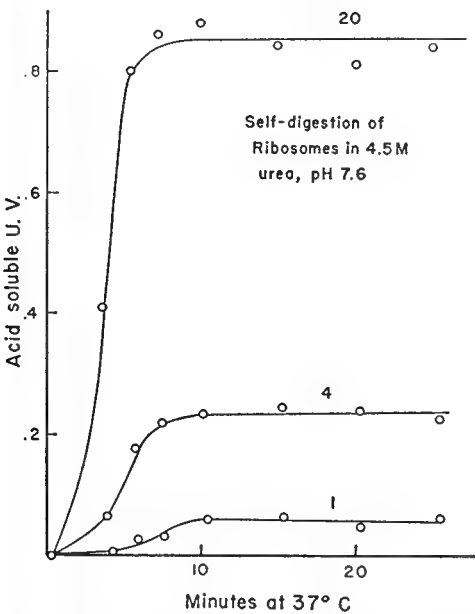


Fig. 26. Digestion of ribosomal RNA by the latent ribonuclease in the ribosomes. Digestion is indicated by the appearance of acid-soluble ultraviolet-absorbing components. The results for three concentrations (1, 4, and 20 arbitrary units) are illustrated.

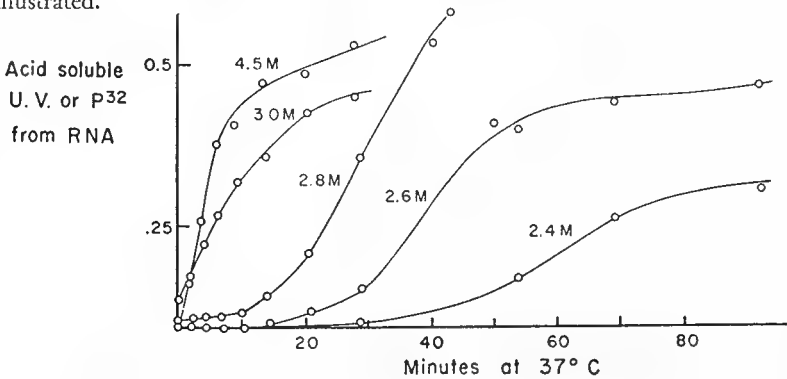


Fig. 27. The effect of urea concentration on the self-digestion of ribosomes. Urea concentrations of 2.4 to 4.5 M were used.

Attempts to determine this proportion accurately have been frustrating; our analyses indicate that there may be one ribonuclease molecule for each ribosomal particle as an upper limit, or that there may be ten ribosomal particles for each enzyme molecule as a lower limit. Until an accurate determination can be made, the important question whether ribonuclease is a component of *all* ribosomes must remain open. The enzyme is a component of some, at least, of the ribosomes, however, and it was of interest to inquire into the basis of its latent behavior.

For this purpose ribosomes were suspended in various concentrations of urea at constant low ionic strength, or in solutions of 2.0 M urea with varying ionic strength, and the self-digestion of the inherent ribosomal RNA was measured by the appearance of acid-soluble components.

The results are shown in figures 27 and 28. In the lower urea concentrations, or in the lower salt concentrations in 2.0 M urea, a considerable time lag is observed before digestion takes place. When digestion is initiated it appears to follow an autocatalytic course.

Both urea and EDTA decompose ribosomes, the former by breaking hydrogen bonds and the latter by chelating magnesium. Thus, both hydrogen bonds and magnesium function to hold the ribosomes together. The influence of NaCl in the decomposition of the ribosomes in 2.0 M urea (fig. 28) is most likely a result of

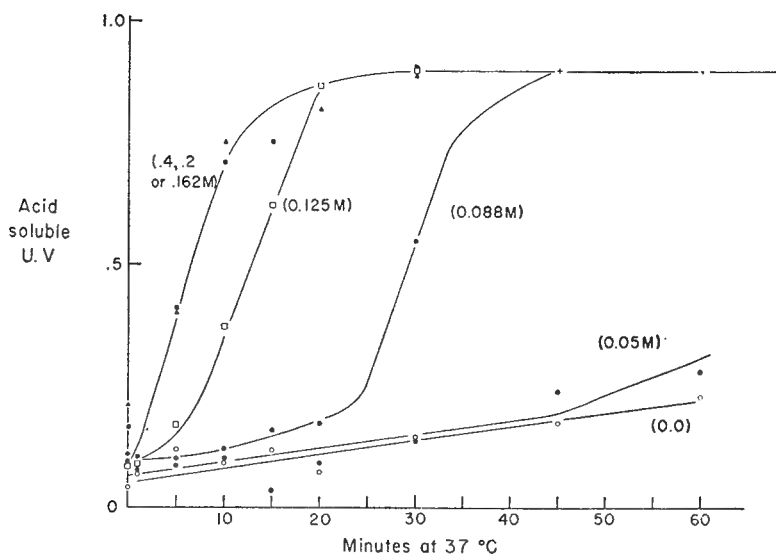


Fig. 28. The effect of NaCl concentration on the self-digestion of ribosomes in 2.0 M urea. The NaCl concentration varied from 0.0 to 0.4 M.

the displacement of magnesium which has been made available to competition as a result of opening some hydrogen bonds. In the absence of urea, NaCl does not bring about ribosome self-digestion. The autocatalytic shape of the self-digestion curves thus appears to result from the loosening of the ribosomal structure until the enzyme is allowed to attack the RNA of a different particle and thereby release more enzymes.

The RNAase of the ribosomes is not completely freed of the ribosomal structure until digestion is complete. This was demonstrated by extracting the enzyme into acetate buffer after ribosomes had been precipitated with trichloroacetic acid over the course of an experiment in which self-digestion was allowed to take place. During the lag period (2.6 M urea, 37° C, TSM buffer) no enzyme was extracted. As digestion occurred ribonuclease activity

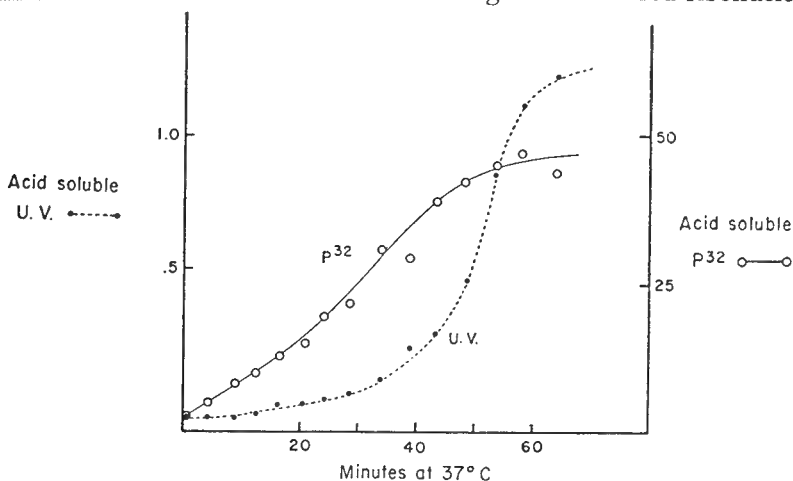


Fig. 29. Digestion of radioactive added RNA and of nonradioactive inherent ribosomal RNA in 2.6 M urea. The former is shown by the appearance of acid-soluble P^{32} , and the latter by acid-soluble ultraviolet-absorbing components.

appeared in the extract. Thus, the ribonuclease is held in the particle in the form of a TCA-precipitable nonacetate-extractable complex until the particle is thoroughly disrupted, at which time it behaves as a "free" molecule.

The interesting question of the intraparticle location of the enzyme was examined in the following way: Nonradioactive ribosomes were treated to provide a lag period before self-digestion took place. Self-digestion was measured by the appearance of acid-soluble ultraviolet-absorbing material. At zero time P^{32} -labeled RNA of high specific radioactivity was added. The digestion of the added substrate was measured by the appearance of P^{32} in the

acid-soluble fraction. The result is shown in figure 29. It is clear that the added RNA is largely digested before the inherent RNA is attacked. Ribosomes that have not been urea-treated do not attack either substrate. Thus, the latency of some, at least, of the enzyme is very rapidly destroyed. It would appear that this latency is due to the existence of hydrogen bonds which keep the enzyme from acting. In addition, the ribonuclease of ribosomes is so "located" that after partial degradation of the particle it is able to attack a high-molecular-weight exogenous substrate but it is not free as shown by the TCA-acetate procedure and is still unable to attack the RNA of the particle.

Comment. Elson and Tal (Biochim. et Biophys. Acta, 36, 281, 1959) and also Spahr and Hollingsworth (J. Biol. Chem., 236, 823, 1961) have presented evidence that the latent ribonuclease resides entirely in the 30S particle. We can find no reason to doubt our finding that the enzyme exists in a latent form in both 30S and 50S ribosomes of E. coli. This problem remains, as does the far more significant one: what is the biological function of latent ribonuclease?

Ellis T. Bolton.

III.D.3 Leucine Aminopeptidase

(Reprinted from Carnegie Institution of Washington Year Book 58, pp. 278-280, 1959.)

E. T. Bolton and B. J. McCarthy

Leucine aminopeptidase. Unlike ribonuclease activity the LAP activity of E. coli shows no latency. It appears fully active toward several leucyl substrates provided that the magnesium concentration is adequate. The ribosomal association of this enzymic function was sought after it had been learned from Dr. A. T. Matheson, of McGill University, that LAP activity was found in a ribonucleoprotein fraction from autolyzed mammalian tissues. Crude ribosome preparations from E. coli invariably showed LAP activity against leucyl amino acid peptides. In some preparations the activity was as small as 10 per cent of the total in the cell,

and in others as high as 80 per cent. The distribution between ribosomes and the soluble fraction apparently depended on the physiological state of the cells when they were harvested. For example, the ribosomes of resting cells which had been grown in a medium rich in glucose showed a greater fraction of the total LAP activities than those from cells grown in a low-glucose medium. The mere presence of enzyme activity in the soluble fraction raised the serious question whether the LAP activity of the ribosomes was an inherent capacity or one brought about as a result of adsorption.

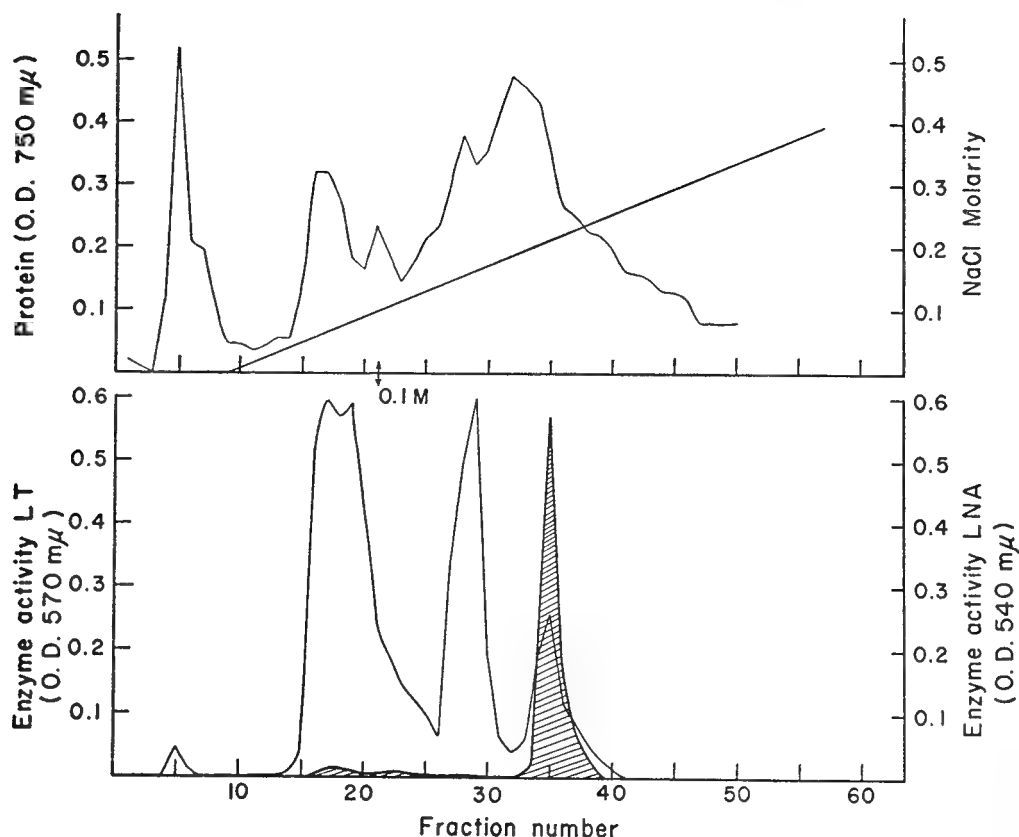


Fig. 30. DEAE-cellulose chromatography of leucine aminopeptidases of the soluble fraction of *E. coli*. The protein content and eluting salt concentration are shown on the upper diagram, and the enzyme activity on the lower one. The activity against leucyltyrosine (LT) is indicated by the unshaded regions; that against leucyl- β -naphthylamine (LNA) is shaded.

Several lines of evidence add up to show that some of the LAP activity of ribosomes is indeed an inherent capacity. When the chromogenic substrate leucyl- β -naphthylamine was used in place of leucyltyrosine to carry out the assays, the ribosomes were found to be devoid of activity, all the enzymic function residing in the soluble fraction. Thus, at least two enzymes capable of splitting leucyl peptides existed. One was characteristically a soluble component. It seems unlikely that ribosomes would specifically exclude one LAP function and retain another, were adsorption responsible. A further test was made by analyzing the LAP activity of purified 30S and 50S particles. These were derived from an original 70 to 100S

mixture and had been through five cycles of differential ultracentrifugation, being finally purified by the swinging-bucket sucrose-gradient method. The results of the analysis are given in table 16.

TABLE 16. LAP Activity of 50S and 30S Particles

Particle	Protein, $\mu\text{g/ml}$	Leucine Liberated, $\mu\text{M/ml/min}$	Enzyme Activity per Unit Protein
50S	1718	0.220	1.26
30S	445	0.059	1.29

It may be observed from these results that the enzyme activity per unit protein

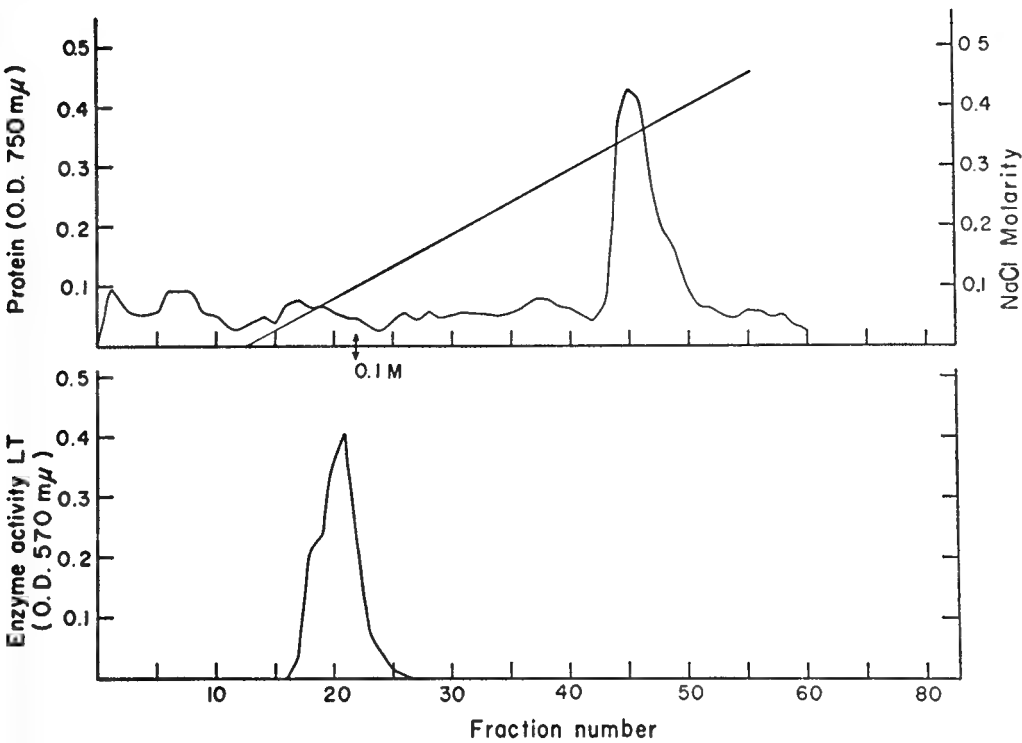


Fig. 31. DEAE-cellulose chromatography of the leucine aminopeptidase of *E. coli* ribosomes. Protein and eluting salt concentration are shown on the upper diagram, and enzyme activity against leucyltyrosine (LT) is indicated on the lower one.

is the same for the two particle types. Again, it seems unlikely that adsorption could account for such a distribution of enzyme activity and its persistence with the particles throughout the involved purification procedure. In still another test DEAE-cellulose chromatography was employed. Ribosomes and also the soluble fraction remaining were chromatographed and all the fractions analyzed for LAP activity against leucyltyrosine. The results are shown in figures 30 and 31. The soluble fraction contained at least three separable LAP activities. The ribosome chromatogram contained only one region which showed the function. The enzyme activity in this region corresponded in chromatographic behavior and also in substrate specificity (table 17) to one of the soluble components. Thus, the ribosomes

TABLE 17. Specificity of Eluted Enzyme Peaks

	Soluble Enzyme Peaks			Ribo- some Peak
	A	B	C	
DL-Alanyl-DL-alanine	—	+	—	—
Glycyl-DL-alanine	—	+	+	—
Glycyl-L-leucine	—	+	+	—
Glycyl-DL-valine	—	+	+	—
L-Leucyl-L-tyrosine	+	+	+	+
L-Leucyl-L-glycine	+	+	+	+
L-Leucylglycyl glycine	+	+	+	+
L-Leucinamide	+	—	+	+
L-Leucyl-β-naphthyl- amide hydrochloride	—	—	+	—
L-Prolylglycine	—	+	—	—

appear to contain only one of a possible three LAP activities. Centrifugal analysis

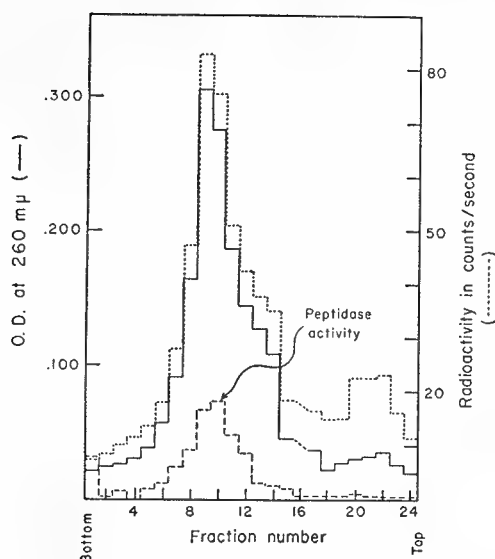


Fig. 24. Swinging-bucket sedimentation analysis of steady-state S^{35} -labeled *E. coli* ribosomes. Optical density at 260 μ indicates RNA content, radioactivity indicates protein content, and enzyme activity indicates leucine aminopeptidase content. The particle peak at fraction 10 is comprised principally of the 70 and 85S components.

of ribosomal particles by the swinging-bucket technique has shown that the enzyme activity tracks upon the particle distribution during sedimentation yielding essentially constant specific enzyme activities across the pattern (fig. 24). Thus, the sedimentation constant for this bacterial LAP activity can be very large, of the order of 70 to 100S. This is in contrast to that for a mammalian LAP which has a sedimentation coefficient of approximately 15S. The ribosomal LAP is dissociated from nucleoprotein by DEAE-cellulose chromatography, as figure 30 shows. The chromatographically separated LAP shows a small sedimentation constant (less than 20S). The yield of LAP by this procedure is disappointingly small (~ 10 per cent of that in the ribosomes), most of the enzyme remaining irreversibly bound to the column. In this respect ribosomal LAP differs from ribonuclease which chromatographs with the nucleoprotein (A peak) on the column. Thus, a different location in the ribosome for LAP and ribonuclease may be inferred.

III.D.4 Composition of the RNA

(Reprinted from Carnegie Institution of Washington Year Book 58, pp. 274-275, 1959.)

E. T. Bolton

Composition of the RNA. The nucleotide compositions of several classes of *E. coli* RNA have been determined by means of isotope dilution, using chromatographic and electrophoretic separations. The results are presented in table 15. C, A, G, and U signify cytidylic, adenylic, guanylic, and uridylic acids, which are the principal building blocks of RNA. S is the RNA which is found in the supernatant fluid after a cell juice has been centrifuged (40K 180'). This RNA is eluted on DEAE-cellulose in region B. CA-B is an RNA produced by cells which have been cultured for $2\frac{1}{2}$ hours in a medium containing 20 μ g/ml of chloramphenicol. This

RNA is also eluted from DEAE cellulose in region B. CA-D is RNA, produced in the presence of chloramphenicol, which is eluted in region D; 30(n) and 50(n) are the RNA's of the 30S and 50S particles which normally occur in juices of exponentially growing bacteria; 30(70) and 50(70) are the RNA's of 30S and 50S particles which were derived from 70S particles by dissociation of the latter in a solution low in magnesium. It is evident from the results in table 15 that *E. coli* RNA may be classified according to composition: one class, the "soluble" type, is characterized by a richness of cytidylic acid and a deficiency in adenylic acid; an-

TABLE 15. Composition of *E. coli* Ribonucleic Acids

	Type of RNA, mole per cent *						
	Soluble			Particle			
	S	CA-B	CA-D	30(<i>n</i>)	30(70)	50(<i>n</i>)	50(70)
C	29.1	27.2	20.2	22.2	23.6	21.0	20.5
A	19.7	19.9	28.6	24.2	24.3	26.4	26.4
G	34.2	35.6	32.2	30.4	31.6	34.1	34.8
U	17.2	17.7	19.0	[23.1]	20.5	18.5	18.3
A+C G+U	0.95	0.89	0.96	0.87	0.92	0.90	0.88

* Values are the arithmetic means of several determinations for each component. The uncertainty is ± 3 per cent of the given value except for the bracketed value where the uncertainty was ± 7 per cent.

other, the "particle" type, is characterized by relatively more adenylic than cytidylic acid. It is also evident that the natural 30S RNA has a composition very similar to that of the 30S material derived from the 70S particle. A similar result obtains for the 50S components. The RNA composition of the 30S particle differs significantly from that of the 50S, however, the 50S particle being richer in purine nucleotides. Thus, it cannot be a simple dimer of 30S particles. The 70S particle, however, is a dissociable polymer, and it could be "synthesized" as a result of association of natural 30S and 50S units. It would appear that the 30S and 50S are "fundamental" particles, the other species, 70 to 100S, being derivatives.

The results for the CA-RNA's are also of interest in the problem of nucleoprotein biosynthesis, since these are RNA's produced in the virtual absence of protein synthesis. CA-B is clearly of the soluble type and is probably not associated with protein. The composition of CA-D, on the other hand, is like that of the nucleoprotein RNA. Thus, particle-type RNA can be made when protein synthesis is suppressed. In addition, the CA-D elutes in the region in which the polynucleotide

precursor of nucleoprotein is found. CA-D has a small sedimentation coefficient (less than 20S), however, whereas the polynucleotide precursor sediments rapidly (20 to 100S).

The DNA of *E. coli* contains nearly equimolar proportions of its component nucleotides, and the bases are paired in the arrangement G:C and A:T. Neither of these conditions is evidenced by the analyses of RNA. Thus, there is no simple correspondence between the composition of DNA and that of any of the RNA's examined. On the other hand, there is a high consistency in the proportions of 6-amino:6-keto groups, as shown by the ratio A+C:G+U in table 15. Thus, the RNA composition seems to be under some restraint. In addition, if it is assumed that all the pyrimidine residues are base-paired (i.e., G:C and A:U), and the excess purine in the RNA's is computed, it is found that the 50S material has roughly twice the excess found for the 30S RNA. Again, there seems to be a kind of systematics which hints at an underlying regulating mechanism. Many more data are needed, however, before more than vague generalizations can be expressed about the determinants of RNA composition.

Comment. It has been commented (P. F. Spahr and A. Tissieres, *J. Mol. Biol.*, **1**, 237, 1959) that "The nucleotide composition of the four principal nucleotides is the same in the three different kinds of ribonucleoprotein particles from *E. coli*, with sedimentation constants of 30S, 50S, and 70S." It was also

stated in the same report, "The composition of the ribonucleic acid appears to be the same in the three different particles, with the possible exception of the cytidylic acid, which seems slightly lower in the 50S than in the 30S particles." Midgley (Biochim. et Biophys. Acta, **61**, 513-525, 1962) has confirmed the analyses presented above (which, incidentally, agree remarkably well with those of Spahr and Tissieres) and shows consistent differences between the 30S and 50S components. By means of nucleotide "fingerprinting," Aronson (J. Mol. Biol., **5**, 453, 1962) has also demonstrated nucleotide sequence differences between the two kinds of particles. Ellis T. Bolton.

III.D.5 Particles of Other Microorganisms

(Reprinted from Carnegie Institution of Washington Year Book **58**, pp. 281-282, 1959.)

B. J. McCarthy

A survey of the particle contents of representative microorganisms suggests that the results of the study of the ribosomal particles of *E. coli* may be of general applicability. Although the study was somewhat cursory, and no attempt was made to estimate accurate sedimentation constants, the general impression gained from ultracentrifuge analysis was one of remarkable uniformity. The organisms studied include six strains of bacteria, two yeasts, and one mold. The more rapidly growing bacteria appeared to have particle contents similar to those of *E. coli*, and at the other extreme *Aspergillus niger* mycelia have very few.

In juices from growing cells particles of 70 to 80S are always predominant. They are usually accompanied by much smaller quantities of one more rapidly and two more slowly sedimenting components corresponding to the 100, 50, and 30S peaks of *E. coli*. There are, of course, differences in the proportions of the various particle sizes from one organism to another. In particular, juices from *Pseudomonas fluorescens* appear to contain relatively large amounts of the smallest particle (~20S). In all organisms studied the stability of

the larger particles seems to be magnesium-dependent, so that reduction of the Mg^{++} ion concentration to 10^{-4} M leaves only the 50 and 30S particles.

Studies of other properties such as column behavior and enzyme content are also of value in comparing particles of different origins. The behavior of particles of *E. coli*, *Aerobacter aerogenes*, *Ps. fluorescens*, *B. megatherium*, and *Salmonella typhimurium* on columns of DEAE appears to be very similar. The fact that all these particles are eluted at the same salt concentration suggests that their structures have much in common.

Tests on highly purified particles from *Ps. fluorescens*, *B. megatherium*, and *A. aerogenes* showed the presence of both RNAase and peptidase activities. Self-digestion of the particle RNA by RNAase appeared to take place under the same conditions as described for *E. coli*, but no detailed comparison was attempted. The specificity of the peptidase reaction of these particles very closely resembles that of the *E. coli* ribosomes. Though there was evidence of RNAase activity in yeast ribosomes, no peptidase activities could be detected.

Comment. In the years following this brief survey many data have accumulated relating to the presence and properties of ribosomes in many different organisms. For a recent comparative account the reader is referred to a review by Ts'o (Ann. Rev. Plant Physiol., **13**, 45, 1962). Latent RNase in ribosomes has been demonstrated in pea seedlings (S. Matsushita and F. Ibuki, Biochim. Biophys. Acta, **40**, 358, 1960) and in yeast (Y. Ohtaka and K. Uchida, ibid., **76**, 94, 1963). Brian J. McCarthy.

III.D.6 Nucleotide Sequences in *E. coli* Ribonucleic Acids

(Reprinted from *Carnegie Institution of Washington Year Book* 59, pp. 260-264, 1960.)

E. T. Bolton

*Nucleotide Sequences in E. coli
Ribonucleic Acids*

The hypothesis that ribonucleic acids may serve as templates upon which amino acids are assembled during the synthesis of protein molecules is a central idea of molecular biology. The sequences of nucleotides in the ribonucleic acids are believed to specify the sequences of amino acids in proteins. Moreover, the constant proportions of protein and nucleic acid in ribosomes indicate a definite arrangement of these parts. Thus, knowledge of the nucleotide sequences of RNA could contribute toward understanding of both the structure and the function of ribosomes.

Currently developing analytical methods make it possible to determine many nucleotide sequences in RNA. Some results of our studies are reported below.

Kinetics of ribosome self-digestion. Ribosomes treated with 4.5 M urea or suspended in a solution of EDT (versene, ethylene diamine tetraacetal) rapidly deteriorate and release a latent ribonuclease which digests the ribosomal RNA. Figure 42(a) shows the release of acid-soluble ultraviolet-light-absorbing components as a function of time after treatment of ribosomes with EDT. The lag shown was purposely introduced by allowing the digest to warm from room temperature to 37°C. During the course of this experiment samples of the acid-soluble fraction were analyzed by the orcinol reaction for purine-associated ribose and also by paper chromatography. The ammonium

sulfate-phosphate buffer-isopropanol chromatographic solvent separates the mixed oligonucleotides, the 2',3'-adenylic acids, the 2',3'-guanylic acids, and the mixed pyrimidine mononucleotides in the order of increasing rate of migration. The results of analyses of each of these by ultraviolet absorption are shown in figure 42(b).

This experiment illustrates some features of the behavior of the bacterial enzyme. The enzymic attack initially produces oligonucleotides more rapidly than it produces mononucleotides. The rate of mononucleotide production is roughly proportional to the oligonucleotide concentration. Thus, oligonucleotides are the precursors of mononucleotides. The enzyme does not appear to degrade the original polynucleotide chains from one end, thereby releasing mononucleotides one by one. Instead, it appears to cleave the large units into smaller ones, and eventually into mononucleotides. All four mononucleotides appear, even at early times. Thus, most of the possible kinds of phosphodiester linkages that make up the backbone of the ribosomal RNA can be hydrolyzed by the bacterial enzyme. The paper chromatograms show that the purine mononucleotides are essentially all of the type in which the phosphate group is esterified at the 3'-hydroxyl of ribose. Even at the end of an hour of reaction, when the RNA is completely acid soluble, about one-fifth of the total residues persist as oligonucleotides. There are, therefore, some linkages which are slowly, if at all,

hydrolyzed. The production of acid-soluble purine-associated ribose (fig. 42*a*) is coincident with the production of acid-soluble ultraviolet-absorbing materials. Hence purine nucleotides are released at about the same rate as other nucleotides.

materials were eluted with KHCO_3 in a concentration gradient as shown by the broken line. Fractions were collected and analyzed.

The shaded peak has no affinity for the cellulose; it results principally from light

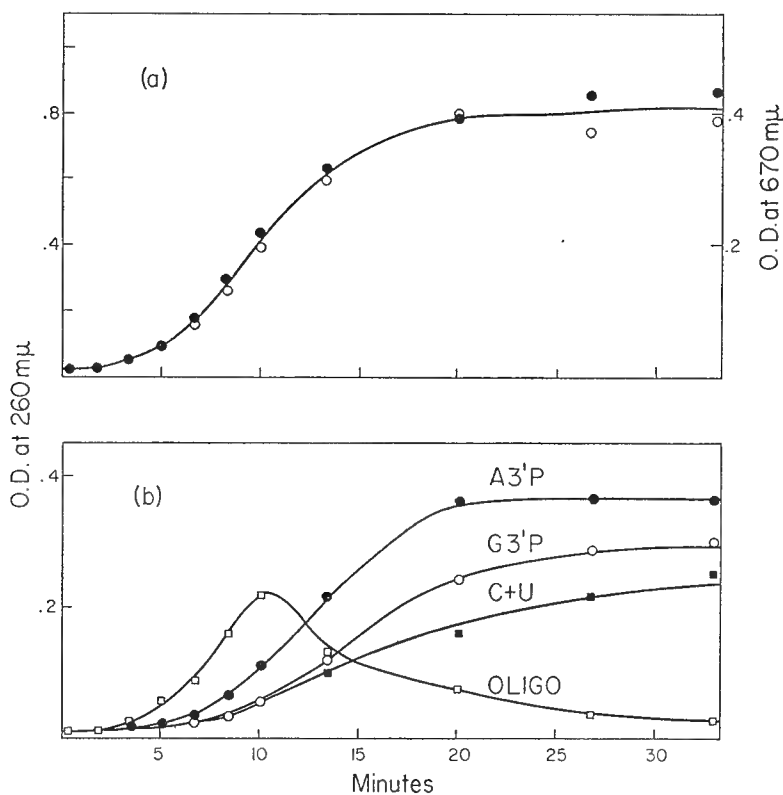


Fig. 42. EDT-induced self-digestion of *E. coli* ribosomes. (a) Release of acid-soluble, UV-absorbing, and orcinol-reactive components. (b) Release of acid-soluble oligonucleotides (OLIGO); adenylic acid (A3' P); guanylic acid (G3' P); and the mixed pyrimidine mononucleotides cytidylic and uridylic acids (C and U). 70S ribosomes were added to EDT (0.05 *M* final concentration), pH 7.6. Aliquots were withdrawn and treated with formic acid to stop the digestion. Samples of the mixture were then chromatographed and analyzed.

Ion exchange of the nucleotide products of self-digestion. Nucleotides may be separated by ion exchange on DEAE-cellulose. An example illustrating the complexity among the oligonucleotide products of self-digestion is shown in figure 43. For this run ribosomes were suspended in 4.5 *M* urea in TSM 5×10^{-3} for an hour at 37°. This preparation was diluted with 5 volumes of water and immediately adsorbed onto DEAE-cellulose. The adsorbed

scattering by denatured ribosomal protein. The first major group to be eluted contains the mononucleotides, all the uridylic acid, about 85 per cent of the purine nucleotides, and a little over half of the cytidylic acid. The sum of the mononucleotides was 83 per cent of the total. In other experiments this value has been as low as 60 per cent and as high as 85 per cent for a 1-hour digestion time. Several of the major peaks containing oligonucleotides were concen-

trated, and a qualitative determination of their nucleotide components was made. The approximate percentage of the total is indicated for each region, together with the nucleotide diagnosis. No uridylic acid was found in the oligonucleotides; adenylic acid (A) was not associated with guanylic acid (G) except for the very last peak, which is undoubtedly a complex mixture; A or G was always associated with cytidylic acid (C); cytidylic acid was also the sole component of two of the peaks. Hence the bacterial enzyme ap-

above agree reasonably well with this calculation they are considered to support the idea that ribosomal RNA is comprised of essentially random arrangements of nucleotides.

Ion-exchange chromatography of beef pancreatic ribonuclease digests. Beef pancreatic ribonuclease digests RNA to yield products of the type $(Pu)_n$ Pyr. (Pu) indicates either or both adenylic and guanylic acids, and Pyr indicates either cytidylic or uridylic acid. These products may be separated by DEAE-cellulose

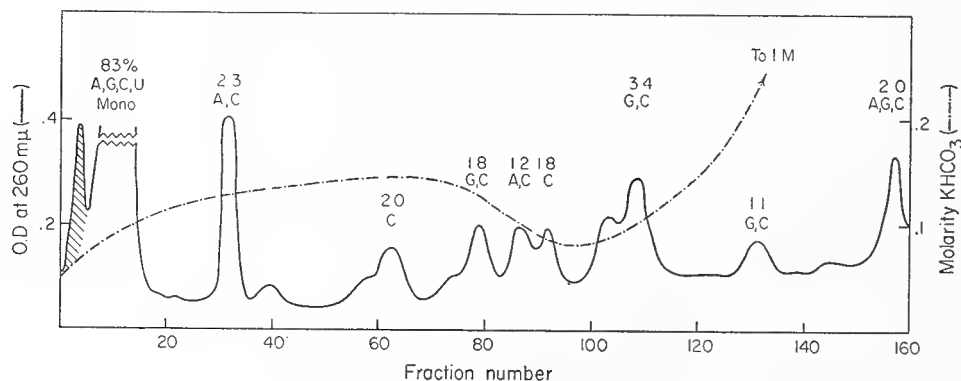


Fig. 43. Elution diagram of a 1-hour self-digest of *E. coli* ribosomes: 70S ribosomes were treated with 4.5 M urea, pH 7.6, for 1 hour. The digest was adsorbed to DEAE-cellulose (1×15 cm column equilibrated with 0.01 M KHCO_3). A KHCO_3 concentration gradient was used to elute the UV-absorbing components. Each fraction contained 4.5 ml. A, G, C, U indicate the residues adenylic, guanylic, cytidylic, and uridylic acids. The numerical values are moles of residues per 100 moles loaded onto the column. Recovery was greater than 95 per cent.

pears to be able to hydrolyze most of the internucleotide linkages rapidly, but the associations of A, G, or C with C are relatively slowly attacked. Preliminary analyses indicate that the oligonucleotides are of the types C_n , or AC_n , or GC_n . If it is assumed that all such oligonucleotides exist at the end of an hour, that their frequency of occurrence is random, and that all the other possible arrangements of nucleotides may be reduced to mononucleotides, then it may be calculated from the known nucleotide composition that all the uridylic acid, 89 per cent of the purine nucleotides, and 50 per cent of the cytidylic acid would appear as mononucleotides. Since the actual findings stated

chromatography. Figure 44 illustrates a separation achieved with a KHCO_3 gradient. For this particular experiment non-radioactive soluble RNA was mixed with P^{32} -labeled RNA extracted from 50S particles. The mixture was treated with beef ribonuclease at 37° for 18 hours. The ultra-violet absorption (O.D. 260 mμ) measures the amounts of components from the S-RNA, and radioactivity indicates the components from the particle RNA. It is evident that many components are common to the two nucleic acids; however, these "fingerprints" do not exactly match. Some sequences exist in the S-RNA (arrows) that are absent or greatly reduced in the RNA_{50} . These sequences in S-RNA

probably contain the uncommon bases (methylpurine, 5-ribosyl uracil, etc.) which have been reported to occur.

The known specificity of the pancreatic enzyme, the known composition of the bacterial RNA's, and the high resolving power of the ion-exchange method make it possible to determine whether the various RNA's are comprised of random arrangements of nucleotides. Our analyses are by no means complete, but they already indicate that the RNA's derived from 70,

nucleotides, eight trinucleotides, and so on.

A digest of RNA₇₀ was chromatographed on DEAE-cellulose using the volatile salt ammonium bicarbonate as the eluting agent. The elution pattern was roughly like that shown for the RNA₅₀ in figure 44. Appropriate fractions were pooled, concentrated, and further fractionated by high-voltage paper electrophoresis at pH 3.5. The compounds resolved in this way were eluted and digested with hydrochloric acid. The products of

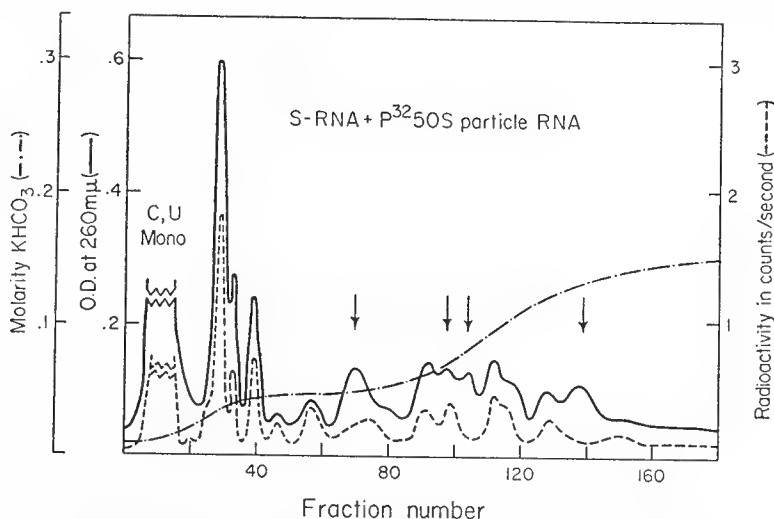


Fig. 44. Elution diagram of a mixture of S-RNA and P³²-labeled RNA₅₀ exhaustively digested with beef pancreatic ribonuclease. O.D. 260 mμ indicates the products from S-RNA and radioactivity from RNA₅₀. The arrows indicate the regions of major differences between the two types of RNA. KHCO₃ gradient elution was employed; 4.5-ml fractions were collected. Recovery was 98 per cent for both S-RNA and RNA₅₀.

50, or 30S particles are probably comprised of all possible sequences and that these occur in the proportions expected from a random association. Using the nucleotide composition of the RNA and applying the "rule," (Pu)_n Pyr, of the beef enzyme, the proportions of mono-, di-, tri-, etc., nucleotides to be expected from random association may be calculated. Expressed as moles of mononucleotide residues per 100 moles, the proportions for RNA₇₀ are: (1) 17.0, (2) 20.2, (3) 18, (4) 14.3, (5) 10.5, (6) 7.6, (7) 5.2, (8) 3.5, (9) 2.3, (10) 1.6 There are expected two kinds of pyrimidine mononucleotides, four kinds of di-

this digest (purine bases and pyrimidine nucleotides) were separated electrophoretically and quantitatively analyzed. By these means the following components of the ribonuclease digest have been found: the two pyrimidine mononucleotides, AAC, AAU, AGC, GAC, AGU, GAU. The other two trinucleotides, GGC and GGU, were probably present, although the poor resolution between these and the still larger chains precluded their identification. Precise measurements of the proportions among the products isolated are not yet available. However, approximately equal amounts (as mononucleotide resi-

dues) of the mono, di, and trinucleotides were recovered, and their sum accounts for about half of the total. For a random arrangement the expected proportions are 17.0, 20.2, and 18.0, and their sum 55.2 per cent of the total. In addition, approximately equal amounts of the four dinucleotides were recovered: the expected proportions are, AC 4.6, AU 4.1, GC 6.0, GU 5.4 per cent. Approximately equal amounts of the pyrimidine mononucleotides were also recovered: for C, 9 mole per cent, and for U, 8 mole per cent, are expected.

Applying the same kinds of calculations for the RNA₅₀ reveals that 16.0 mole per cent of the pyrimidine residues should appear in the pancreatic ribonuclease digests as mononucleotides and their proportions should be C, 8.5, and U, 7.5. By using P³²-labeled RNA₅₀ and separating the mononucleotides electrophoretically 15 mole per cent was recovered from the pancreatic ribonuclease digest as pyrimidine mononucleotides, and these were in the proportions C, 7.6, and U, 7.4.

More than 90 per cent of the digested nucleic acid is recovered from the ion exchanger. Since about half the residues are recovered as trinucleotides or smaller elements, the remainder must exist as tetranucleotide and larger structures. By analogy with the chromatographic behav-

ior of synthetic polyadenylic acids of known chain length it appears probable that the digests of the bacterial RNA contain oligonucleotides of at least 8 residues. Since the longer chains are so infrequent it will probably not be possible to determine directly whether all the possible variants exist, although the separation and identification of the 16 possible tetranucleotides could be accomplished with present methods.

These studies support the idea that the RNA's of *E. coli* are comprised of nucleotide sequences that occur with random frequency. The minimum number of residues in a single chain from RNA₇₀ that will permit a recovery of 17 mole per cent of pyrimidine mononucleotide is 133, if it is assumed that all the chains are alike and that all 8 trinucleotides are represented once. Such a chain would permit the existence of only a *single* 7-nucleotide sequence. All longer sequences would be excluded. To accommodate all 16 possible tetranucleotides the chain would necessarily be composed of 450 residues, a molecule unlikely to have a sedimentation constant of 4S. Therefore, it is apparent that the RNA molecules must be of many different kinds, differing in the sequences in which the nucleotides are arranged.

III.D.7 Studies with *Tetrahymena*¹

(Reprinted from Carnegie Institution of Washington Year Book 59, pp. 279-285, 1960.)

R. J. Britten

STUDIES WITH *Tetrahymena*¹

The central problem in studies of macromolecular biosynthesis may be briefly phrased in the following questions. Does the information stored in the DNA of the cell specify all the properties of the macromolecules? If so, by what mechanism is the information made available at the actual sites of synthesis? With the demonstration that the synthesis of protein molecules is carried out on the ribosomes it seems more likely than ever that RNA is an intermediate in the information transfer. If this is so, at least some fraction of the RNA must be synthesized in association with DNA. The successful observation of nascent (newly synthesized) protein associated with the ribosomes suggests that a search for nascent RNA in association with DNA might be fruitful. If this hypothetical nascent RNA could be observed and identified with ribosomal RNA or some other type of RNA, models of the process of information transfer could be constructed on a sound experimental basis.

There are no adequate guides for a search for nascent RNA. The type of association with the DNA, the amount of the complex, and its stability during extraction procedures cannot be predicted. It would appear necessary, however, to work with the mildest possible methods of extraction and separation of the cellu-

lar RNA and DNA. Unfortunately, all cells contain both DNAase and RNAase. Further, in bacteria the condition for inactivation of the DNAase by removal of Mg^{++} ions is precisely the condition for the activation of the latent RNAase of the ribosomes. Exhaustive attempts to solve this bacteria problem have not yet been carried out. Nevertheless, it appeared attractive to study an organism in which the nucleus might be separated from the remainder of the cell as the first step in the analysis.

Initial examination of *Tetrahymena* indicated that it might be possible to prepare the large (10-micron-diameter) macronuclei in good yield. The possibility of studying the kinetics of RNA synthesis in the nucleus was also tempting, as a first step, even though such results would not directly answer the question at hand.

Relatively pure preparations of nuclei in good yield have been obtained, but the procedures are not yet sufficiently reproducible for the studies of the kinetics of RNA synthesis. However, these studies have led to a simple salt extraction method for the preparation of ribonucleoprotein particles and DNA under conditions in which neither of the nuclease activities is evident.

Preliminary tracer studies by this method have not given any evidence of nascent RNA in association with the DNA. However, the labeling time used was fairly long (1 per cent of the generation time). The DNA content of *Tetrahymena* is only 5 per cent of its RNA content, and it can hardly be presumed that a very large fraction of the DNA would at any time have the hypothetical nascent RNA in association with it. As a result very much shorter

¹This work was performed during a year's visit to the Carlsberg Laboratory and the University Institute of Microbiology, Copenhagen, Denmark. The author (R. J. B.) wishes to express his appreciation to Professor Heinz Holter and Professor Ole Maaløe for generous hospitality and valuable discussion.

labeling times will have to be used to make an adequate test of the method.

Description of *Tetrahymena*. *Tetrahymena pyriformis* is a holotrichous pyriform ciliate with dimensions of about 50 by 30 microns. Its nuclear components consist of an approximately spherical macronucleus of about 10-micron diameter, making up roughly 3 per cent of the cell mass, and a micronucleus of about 1- to 2-micron diameter. The macronucleus is indispensable and is presumably concerned with metabolic processes. The micronucleus is involved in sexual processes and is absent in some naturally occurring strains, which do not undergo a sexual cycle. The micronucleus is not observable in the strain (GL) used in this work. In the absence of sexual processes the macronucleus appears simply to divide at or near the time of cell division. Chromosomal bodies have never been observed in the macronucleus or in strains in which the micronucleus is absent.

In its natural fresh-water habitat *Tetrahymena* is a bacteria feeder, but it can be grown on a chemically defined medium containing eleven amino acids, nine growth factors, and two nucleic acid bases (guanine and uracil). This medium is complex but not unmanageably so, and exponential growth with a generation time of 8 to 10 hours can be maintained. The organism is quite suitable for tracer experiments since it will incorporate uracil into RNA (and presumably also into DNA after conversion to cytosine), thymine specifically into DNA, and amino acids into protein. For most of this work the cells were grown in 0.2 per cent peptone, 0.02 per cent liver extract with a generation time of about 7 hours. At this concentration of peptone they will grow exponentially to better than 0.1 mg dry material per milliliter. In growing to this density they take up 20 per cent of the uracil and 3 per cent of the thymine present in the peptone medium. The pool of uracil in cells growing in this medium is not large, and the rate of uptake of C^{14} -

uracil reaches its final value after about 10 minutes (2 per cent of the generation time). Orthophosphate is not a suitable tracer for pulse experiments since there is a delay of several hours before the rate of incorporation reaches its final value.

The organism is quite fragile in comparison with most bacteria. Though this is a disadvantage in washing and harvesting, it may be of considerable advantage in cellular fractionation since the vigorous breakage methods used for *E. coli* may be avoided. However, when the cells are lightly broken (for example, by pipetting through a fine orifice) the ribosomes do not go into solution. There are distinct possibilities for the study of aspects of the organization and synthesis of the ribosomes which are not accessible in bacteria. Electron microscopy indicates that *Tetrahymena* contains an endoplasmic reticulum. *Tetrahymena* has many similarities, both in organization and in biochemistry, to higher animal cells, and it can be grown conveniently on simple media.

Preparation of *Tetrahymena* nuclei. The relatively large size of the macronucleus and its spherical shape with a somewhat granular surface make it easily recognizable in phase microscopy of broken cell preparations. For most of this work, however, the cells were stained with acridine orange, and the preparations were examined under a fluorescence microscope. *Tetrahymena* can be stained in suspension in its growth medium without preliminary fixation, and lightly stained individuals will survive for very long periods of time. Over a wide range of acridine orange concentrations (10^{-6} to 10^{-3} g/ml) the nucleus shows a bright greenish white fluorescence. At very high concentrations it finally turns orange. The cytoplasm exhibits a large number of bright orange (RNA-containing) granules whose intensity of fluorescence and apparent number increase with the degree of staining. The stained nuclei may be easily examined under the fluorescence microscope for size, degree of apparent damage, and adhering orange cytoplasmic fragments.

Up to this time it has not been possible to obtain good yields of nuclei from unstained cells. Apparently the requirements are very critical for preservation of the nuclei of broken cells. But nuclei that have been stained with acridine orange (from cells stained for a few minutes in 1/10,000 acridine orange in 0.2 per cent peptone) have lost their extreme fragility and can be handled in a variety of solutions (even in distilled water). The *Tetrahymena* individuals themselves, stained in the above fashion, however, become quite fragile and some are broken simply by centrifugation at 5000 rpm, particularly if the culture has exceeded the limiting density for exponential growth. Variability is observed in the fragility and degree of staining from day to day, depending apparently on the exact state of the cells; nevertheless, individual preparations are quite uniform.

Of the various means tried for breaking the cells without excessive damage to the nuclei the most convenient and reproducible seems to be the Waring Blendor. If 5 ml of suspension is blended in the micro attachment for about 1 minute (at somewhat less than half speed), all the cells will be broken down to fragments of varying size and practically all the visible nuclei will be freed from cellular debris. Somewhere between 50 and 100 per cent of the nuclei survive.

Two methods have been somewhat successful in separating the nuclei from the cytoplasmic fragments. The simpler one (which was tried toward the end of the work and has not been fully explored) utilizes sedimentation analysis. A sample of the broken cell suspension was floated over a stabilizing gradient (7 ml, 5 to 20 per cent sucrose) and centrifuged at 2000 rpm for about 1 minute. The nuclei sedimented about 4 cm in a broad band, and the small cytoplasmic granules remained at the top. This method worked successfully on preparations that contained no large cell fragments, but it has not been possible to reproduce the breakage well enough to achieve consistently good preparations.

The more fully explored method involves centrifugation toward equilibrium at 40,000 rpm in a very stiff sucrose gradient (45 to 70 per cent). This method is based on the observation that both the cytoplasmic granules, containing the bulk of the RNA, and the large fragments have a low effective density in sucrose solutions and come to rest at sucrose concentrations of about 50 per cent. The nuclei have a large effective density (though still surprisingly low), and come to rest at about 65 per cent sucrose (density 1.3). Since the concentrated sucrose solutions have a high viscosity it was inconvenient to make gradients. The runs were usually made with layers of sucrose solutions (70, 65, 60, 55, and 45 per cent), with a sample of the broken-cell suspension floated on top. After 1 hour of centrifugation at 40,000 rpm in the swinging-bucket rotor at 5°C most of the nuclei were found resting on top of the 70 and 65 per cent layers and a few still associated with cell fragments on the upper layers. The nuclei recovered appeared to be quite free of orange cytoplasmic granules. The green fluorescence was somewhat diminished, but the nuclei could easily be counted and examined for damage. In the best runs 60 per cent of the original nuclei were recovered as purified free nuclei. Occasionally the yield was poor and the nuclei appeared shrunken and distorted, presumably because of lack of control over the stabilization of the nuclei by acridine orange staining.

In one experiment in which the nuclear preparation scheme worked effectively there was some evidence that RNA was synthesized in the nucleus of this organism and moved out to the cytoplasm. For this experiment labeled orthophosphate was added to a culture growing with a 9-hour generation time. After ½ hour a sample ("pulse") was chilled and carrier orthophosphate was added to the remaining culture. At the end of the succeeding ½ hour another sample ("chase") was chilled. Owing to the large pool in *Tetrahymena* the chase was not very effective, and the rate of increase of radioactivity of

the RNA at the time of the chase sample was greater than half of what it was at the time of the pulse sample. For the pulse sample the radioactivity per purified nucleus was 40 per cent of the radioactivity per cell; for the chase sample the radioactivity per purified nucleus was 23 per cent of the radioactivity per cell.

The results of this experiment are consistent with other trials and suggest that RNA is in fact synthesized in the nucleus. For a number of reasons, however, this result cannot be considered established in *Tetrahymena*. For example, the possibility of contamination of the nuclear fraction has not been adequately checked.

It seems likely that the variability can be brought under control and the kinetics of RNA synthesis in the nucleus of *Tetrahymena* can then be studied quantitatively. The synthesis of RNA in the nucleus has been established in several organisms by means of radioautography, but good measurements of the kinetics are still lacking.

Search for nascent RNA associated with DNA. During the studies of *Tetrahymena* it was observed that the nuclei dissolved in strong salt solutions. The process was very rapid for all salts tested (except for lead salts, which will be discussed in a later section). When these microscopic observations were followed up by studies with tracers and sedimentation analysis it was found that both the RNA and DNA could be easily brought into solution by treating previously frozen whole *Tetrahymena* with 2.4 M CsCl. This concentration of CsCl (density 1.3) was chosen because the residual cytoplasmic material and the soluble proteins floated while the RNA and DNA were sedimented in the ultracentrifuge. The observed sedimentation rates indicate that high-molecular-weight DNA and ribonucleoprotein are preserved during the extraction, and are partially separated. Therefore, an attempt was made to observe nascent RNA in association with DNA without a preliminary purification of the nuclei.

For this method of extraction and sedimentation analysis a growing culture (labeled with C^{14} -uracil or -thymine) was chilled, harvested, and washed with the 0.2 per cent peptone growth medium. The cells were finally concentrated into a compact pellet which was weighed, frozen, and thawed to 0°C twice. It was then frozen again, and a quantity of 8 M CsCl was added to bring the density to 1.3 (approximately 2.4 M). Immediately on thawing, the pellet liquefied to a slightly gelatinous suspension with greatly reduced opacity. It was possible to work with such a concentrated cell suspension because of the small quantity of dry matter (about 10 per cent) in *Tetrahymena*. Three-tenths of a milliliter of this suspension was floated on top of a previously chilled linear gradient of CsCl (density from 1.5 to 1.35) prepared with the gradient mixing device.

The system was then centrifuged in the swinging-bucket rotor of the ultracentrifuge. The undissolved fraction of the cell formed a thin scum at the top of the liquid which was pushed against the side of the tube, where it adhered. The fluid contents were then dripped out into a series of tubes, and the pellet and scum were resuspended separately. After appropriate dilution the optical density at 260 m μ was measured, and TCA was then added to 5 per cent. The TCA precipitates were collected on membrane filters, which were dried, counted, and then assayed for DNA by the Burton modification of the diphenylamine reaction. The filters cause a small background color which was eliminated by taking the difference between the optical density at 600 and 675 m μ . Ten micrograms of DNA gave a reading (600–675) of 0.145, and the detectable limit was about $\frac{1}{2}$ μ g of DNA. With the membrane filter method this quantity of DNA could be harvested out of a large volume of liquid and the sensitivity might be increased by more than a factor of 10 using microcuvettes and small reaction volumes.

With 6 hours of centrifugation at 35,000 rpm it was found that the DNA had

moved about halfway down the tube, as shown in figure 64. When cells were analyzed that had been grown in 0.2 per cent peptone to which C^{14} -thymine was added it was found that in all cuts the TCA-precipitable radioactivity was proportional (± 5 per cent) to the amount of DNA as measured by the diphenylamine reaction. The amount of DNA remaining in the scum layer was only 5 per cent of the total. These results show that C^{14} -thymine is taken up specifically into DNA (in this medium) and that there are no TCA-precipitable substances in *Tetrahymena* which interfere with the diphenylamine reaction. Ninety-five per cent of the DNA of whole

Tetrahymena appears in one broad peak with a sedimentation rate at 0°C (in CsCl of mean density 1.4) corresponding to 13.5S. Assuming an effective density for the DNA in CsCl of 1.7, calculation gives a sedimentation constant in water at 20°C of 50S for the center of the DNA peak. The lack of DNA of low sedimentation constant indicates that, if there is any DNAase in this organism, its activity has been suppressed. The calculated sedimentation constant is greater than that observed for DNA from many tissues and is very near the maximum published value (80S). It may be presumed that the DNA prepared by this method has not been seriously degraded.

In several runs the DNA concentration in the peak cuts was $25\text{ }\mu\text{g/ml}$. There was no sign of gel formation, however. In contrast, when $25\text{ }\mu\text{g/ml}$ of T4 bacteriophage DNA (prepared by osmotic shock) was suspended in a similar concentration of CsCl , its presence was obvious from the strong gel that was formed. This observation suggests that the DNA may be in the form of a nucleoprotein comparable to the 50S nongelling nucleoprotein prepared by Doty from thymus tissue.

The bulk of the RNA survives in an object of high sedimentation constant which has just been pelleted in the 6-hour spin shown in figure 64. Shorter spins show the major peak sedimenting (in CsCl of mean density 1.45 at 0°C) at a rate corresponding to 24S. Since RNA with such a high sedimentation constant has not been observed before, it must be presumed that ribosomes have survived. On the assumption that this peak is made up of ribosomes and that they have an effective density of 1.7 in CsCl (as do bacterial ribosomes) the calculated sedimentation constant in water at 20°C is 90S. This result supports the presumption that this peak is made up of ribosomes. The presumption is further supported by the observation that the pellet, when diluted in water in the absence of magnesium, undergoes complete self-digestion. In a few hours, at room tem-

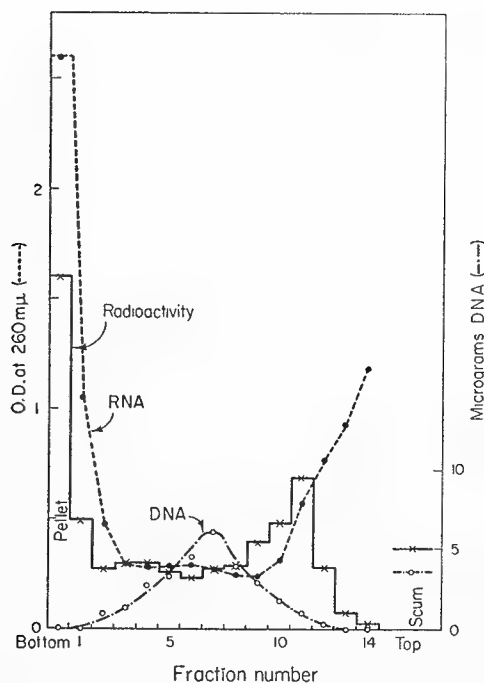


Fig. 64. Sedimentation analysis of *Tetrahymena* nucleic acids after 3 minutes' incorporation of C^{14} -uracil. Centrifugation for 6 hours at 35,000 rpm of a sample of frozen cells treated with 2.4 M CsCl and suspended over a gradient of CsCl (density 1.5 to 1.35). Solid circles, optical density at $260\text{ m}\mu$ measures RNA except in cuts 11, 12, 13, 14, where soluble proteins interfere. Open circles, DNA measured by diphenylamine reaction. Crosses, TCA-precipitable radioactivity derived from C^{14} -uracil.

perature, the TCA-precipitable uracil label falls to about 1 per cent of the initial value. It is implied that ribonuclease is present in the *Tetrahymena* ribosomes as has been clearly demonstrated for bacterial ribosomes. It is fortunate that the ribosomes do not undergo major self-digestion in the CsCl gradient; it may, however, occur to some extent and influence the results shown in figure 64.

In spite of this risk of the expression of ribonuclease activity an attempt has been made to observe nascent RNA in association with the DNA prepared by the above method of extraction and sedimentation analysis. There is a further risk that the concentrated CsCl solution may dissociate the hypothetical complex of nascent RNA and DNA. If there were hybrid base pairing between the RNA and DNA the complex would probably survive, since the transforming activity of bacterial DNA is not affected by banding in 8 M CsCl.

Figure 64 shows the results of an experiment in which a culture of *Tetra-*

hymena growing in 0.2 per cent peptone with a generation time of 7 hours was supplied with C^{14} -uracil for 3 minutes before sudden chilling to 0°C, followed by the procedure described above. There is no evidence of radioactivity associated with the DNA peak. The background of radioactivity due to RNA present in the region of the DNA makes it difficult to set a limit on the possible amount of nascent RNA. A study of the conditions for stability of ribosomes and their size distribution will probably make it possible to reduce this background. Labeling times can be made much shorter by growing the cells in a defined medium with limiting uracil concentration, and this procedure will probably increase the sensitivity.

That the specific radioactivity of the RNA in the region of the DNA and in the peak in fraction 11 is about twice that of the bulk of the RNA in the pellet suggests that, as in bacteria, the small ribosomes are labeled before the larger ones during the course of synthesis.

III.D.8 Specific Yeast RNA Analysis

(Reprinted from Carnegie Institution of Washington Year Book 60, pp. 309-311, 1961.)

J. E. M. Midgley

Specific Yeast RNA Analysis

The enzyme triose phosphate dehydrogenase is produced in large quantity by certain yeasts and accounts for 30 per cent of the total protein. A small fraction of the enzyme is associated with the ribosomes, and antisera to the purified enzyme will precipitate the ribosomal fraction of the enzyme together with 8 per cent of the ribosomes. As these particular ribosomes differ from the rest in their association with the enzyme they might well be "specific" ribosomes which participate in the synthesis of the enzyme. Dr. Goldthwait of Western Reserve University, who has studied this system, sent us samples of the RNA de-

rived from the ribosomes of this yeast together with RNA from the two fractions of the ribosomes separated by precipitation with antisera to the enzyme. These were analyzed to determine whether any differences could be detected in their chemical properties.

All samples of RNA were supplied to us after phenol treatment and alcohol precipitation.

The passage of these RNA's through Sephadex G50 indicated that there was little material of molecular weight less than 10,000. The various fractions were first analyzed for base composition by isotope dilution using P^{32} -labeled RNA from *E.*

TABLE 12. Yeast RNA Composition

	"Non-specific" RNA	"Specific" RNA	Supernatant RNA after Removal of "Specific"
C	18.9 \pm 0.3	19.0 \pm 0.3	18.6 \pm 0.3
A	25.0 \pm 0.4	26.1 \pm 0.4	25.1 \pm 0.4
G	30.6 \pm 0.5	31.1 \pm 0.5	31.1 \pm 0.5
U	25.5 \pm 0.4	23.7 \pm 0.4	25.2 \pm 0.4

coli. The results are indicated in table 12.

It is apparent that the difference in composition of "specific" RNA and the total ribosomal RNA in the cell is only marginal. Adenylic acid appears to be about 4 per cent richer and uridylic acid 8 per cent poorer in the "specific" material as compared with the total RNA. Although these differences are probably real, this experiment by itself was not considered to be a sufficient indicator of the existence of specific RNA's within the total ribosomal material.

Since it had been shown (Year Book 59) that 50S and 30S *coli* RNA are slightly different in their elution from DEAE, it was thought that differences in elution pattern might exist between the yeast "specific" and "nonspecific" ribosomal RNA. Accordingly, yeast RNA (mixed with P³² *coli* RNA as an internal standard) was eluted from a DEAE column by a linear gradient of 0.02 M NaCl/0.01 M tris buffer, pH 7.4, to 0.8 M NaCl/0.05 M tris buffer, pH 8.8. It was thought possible that, as the specific yeast RNA might be more homogeneous than the nonspecific yeast RNA, there might be a greater spread of ultraviolet absorption (compared with the spread of radioactivity of the internal standard) with the nonspecific than with the specific RNA. If the radioactivity at the peak was compared with the radioactivity at a point of half the ultraviolet absorption on the trail of the material, the ratio

$$\frac{\text{Specific radioactivity at peak of UV absorption}}{\text{Specific radioactivity at point of half absorption}}$$

should be higher for the nonspecific than for the specific RNA from yeast, owing to the greater spread of the nonspecific peak. The conditions of elution were made as nearly identical as possible. The ratios observed in two runs of the specific RNA were 1.23 and 1.21; the nonspecific RNA gave ratios of 1.35 and 1.32. Thus the specific RNA has a slightly sharper peak.

To test for any differences in nucleotide sequences (which would not be detected after complete hydrolysis) the specific and the nonspecific yeast RNA's were digested with pancreatic ribonuclease in the presence of P³²-labeled *coli* ribosomal RNA as the internal standard.

After 18 hours at 37°C the digests were absorbed on a DEAE column in 0.02 M ammonium bicarbonate at pH 8.6. The elution of the oligonucleotides was carried out by a linear gradient of 0.02 to 0.5 M ammonium bicarbonate, pH 8.6. At a suitable time, the remaining absorbed material was then eluted with 1 M ammonium bicarbonate. The recovery of material from the column was not less than 96 per cent in any measurement.

Figure 53 shows the oligonucleotide pattern of elution from the DEAE column by the ammonium bicarbonate gradient. It can be seen that cytidylic acid, uridylic acid, and most of the di- and trinucleotides were separated. The materials in the various peaks were identified by comparison with the known elution pattern of the oligonucleotides and also by the changes in absorption spectrum between 240 and 300 mμ from pH 8.6 to pH 2.

The results from partial digestion by pancreatic ribonuclease do not show any qualitative difference. They do, however, reinforce the differences between the specific yeast ribosomal RNA and the nonspecific RNA observed from base composition analysis and elution patterns on DEAE (table 13). It is thus very probable that the RNA precipitated by the rabbit anti-serum from yeast differs marginally from the total RNA in the yeast ribosomes.

It is quite likely that the enzyme-forming unit (70S ribosomes) may include both

specific and nonspecific components. The nonspecific components would obscure any differences that might exist in the specific portions. Fractionation of the antisera-

precipitated 70S particles before analysis might then show one fraction having a convincingly different composition.

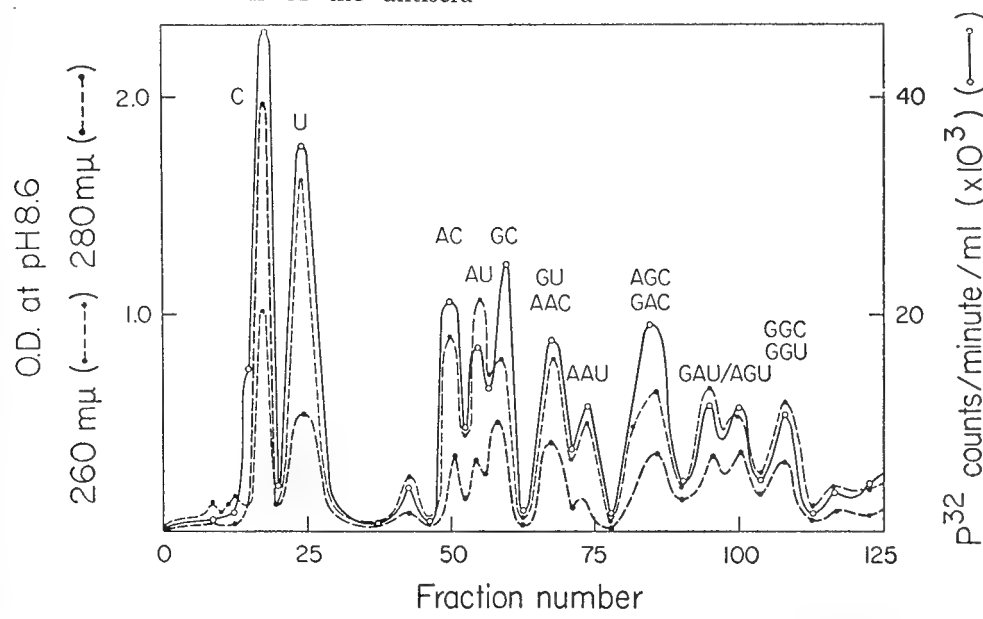


Fig. 53. DEAE-ammonium bicarbonate analysis of pancreatic ribonuclease digestion products of a mixture of P^{32} -labeled *E. coli* B RNA and a large excess of unlabeled yeast "specific" RNA. A linear elution gradient of 0.02 to 0.5 M ammonium bicarbonate, pH 8.6, was used.

TABLE 13. Distribution of Mono- and Dinucleotides

	Nonspecific		Specific	
	Calculated	Observed	Calculated	Observed
Mononucleotide	19.69	19.49	18.2	18.23
C	8.39	8.53	8.17	8.43
U	11.30	10.96	10.12	9.80
Dinucleotide	21.92	20.88
AC	4.19	4.24
AU	5.66	5.29
GC	5.13	5.05
Nonspecific				
Specific	Calculated		Observed	
C	1.027		1.019	
U	1.126		1.103	
AC	0.988		0.991	
AU	1.070		1.050	
GC	1.016		1.027	

Comment. It is now obvious that the ribosomes should have been removed from the preparation before the composition was measured. Richard B. Roberts.

III.D.9 DEAE Chromatography of Ribosomes

(Reprinted from Carnegie Institution of Washington Year Book 60, pp. 328-330, 1961.)

B. J. McCarthy

DEAE Chromatography of Ribosomes

References to the use of DEAE cellulose columns for the chromatography of ribosomes have appeared in the last three Year Books. In particular it has been suggested that under some conditions it is possible to obtain nucleoproteins that have lost a certain fraction of their protein as a result of chromatography. Since chromatography has been used extensively this year to study ribosome synthesis, many of these experiments have been repeated for purposes of clarification.

In the first place, it has been shown that if the magnesium concentration is kept sufficiently high during the elution no diminution of the protein content of the resulting ribosome occurs. Two separate batches of carefully purified ribosomes were studied. 70S ribosomes were selected from a cell extract by a 40-minute centrifugation at 40,000 rpm and purified by means of three further similar centrifugations and resuspensions. Finally the protein:RNA ratio of 70S ribosomes was determined by sedimentation analysis in a sucrose gradient. One sample of ribosomes was made from cells randomly labeled with S^{35} , and the protein:RNA ratio was expressed therefore as the ratio of TCA-precipitable S^{35} cpm to optical density at 260 m μ . For the other sample ribosomes were prepared from cells randomly labeled both with C^{14} -leucine and P^{32} , and the ratio was expressed as TCA-precipitable C^{14} cpm to P^{32} cpm.

A study was then made of the elution pattern resulting from chromatography as a function of magnesium concentration. The ribosomes were always loaded on the column in tris-HCl 0.01 M, pH 7.4, $MgCl_2$ 0.01 M. Elution was carried out by means

of a linear NaCl gradient from zero to 1.0 M NaCl in the tris HCl 0.01 M, pH 7.4, buffer containing $MgCl_2$ at a range of concentrations from 10^{-2} to 10^{-4} M.

Figure 65 shows the elution pattern for the S^{35} -labeled ribosomes in 10^{-2} , 5×10^{-3} , 2.5×10^{-3} , and 10^{-3} M; and table 15 lists the approximate protein:RNA ratios of the various peaks obtained relative to the 70S obtained from the swinging bucket for both ribosome preparations. At 10^{-2} M only a single peak is visible at 0.4 M NaCl, and this has the same protein content as the original ribosomes. These can be shown to be undegraded 50S and 30S ribosomes. At 5×10^{-3} M a second peak appears at about 0.5 M NaCl having only some 25 to 30 per cent of the original protein. It should be emphasized here that quantitative recovery of RNA was obtained throughout these experiments so that the low protein:RNA ratios reflect loss of protein. At lower concentrations of magnesium the 0.5 peak first increases and then decreases as a third peak at 0.6 M NaCl appears. This last component has an estimated protein content of about 5 per cent of the original ribosomes. Very similar results were obtained from the leucine-labeled ribosomes, and it is therefore likely that these differences reflect proportionate losses in protein content rather than the loss of sulfur-rich proteins.

In the kinetic analysis of ribosome synthesis it was shown that the two precursors eluted at 0.6 M and 0.5 M NaCl. The positions and peak shapes of the two precursors coincide very well with the two artificially produced nucleoprotein peaks. It is tempting to believe that the combined effects of low magnesium concentration and the DEAE cellulose column have succeeded in

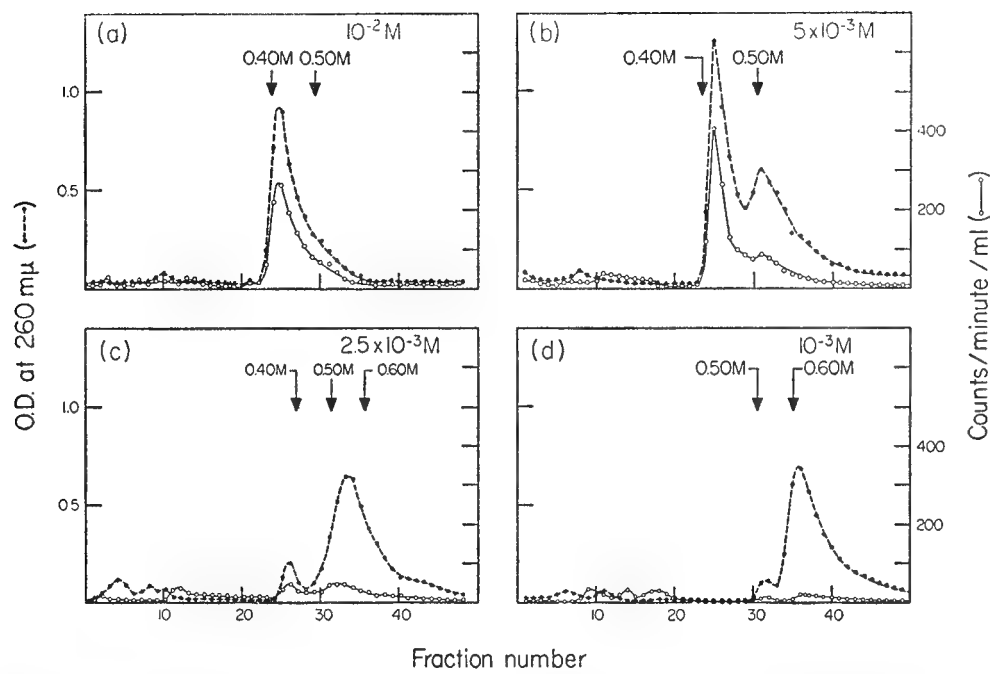


Fig. 65. Chromatography on DEAE cellulose of purified 70S ribosome randomly labeled with S^{35} linear NaCl gradient from zero to 1.0 M in tris-HCl 0.01 M, pH 7.4, containing: (a) 10^{-2} M $MgCl_2$, (b) 5×10^{-3} M $MgCl_2$, (c) 2.5×10^{-3} M $MgCl_2$, (d) 10^{-3} M $MgCl_2$. Salt gradient 0.004 M/ml.

TABLE 15. Effect of Magnesium Concentration on Chromatography of Ribosomes on DEAE Cellulose

During Elution	Concentration of Mg^{++} Protein Content of Nucleoproteins		
	Relative to 70S Ribosomes		
	0.4 M NaCl Peak	0.5 M NaCl Peak	0.6 M NaCl Peak
I. S^{35} -labeled ribosomes			
10^{-2}	0.99		
5×10^{-3}	0.98	0.24	
2.5×10^{-3}	0.84	0.32	0.15
10^{-3}		0.28	0.08
3×10^{-3}		0.24	0.06
10^{-4}			0.05
II. C^{14} -Leucine-labeled ribosomes			
10^{-2}	1.02		
5×10^{-3}	0.98	0.26	
3.7×10^{-3}	1.03	0.23	
2.5×10^{-3}		0.20	0.06
10^{-3}		0.27	0.05

Protein contents expressed as relative to 70S ribosomes purified by sedimentation analysis in a sucrose gradient.

- I. Protein:RNA ratios expressed as S^{35} cpm/O.D. at 260 mμ (cells randomly labeled with S^{35}).
- II. Protein:RNA ratio expressed as C^{14} cpm/ P^{32} cpm (cells randomly labeled with P^{32} and C^{14} -leucine).

degrading ribosomes back to neosomes and eosomes. Further experiments are in progress aimed at measuring the sedimentation coefficients of the 0.5 and 0.6 *M* peaks. The work of Waller and Harris has shown great complexity in ribosome proteins. It may therefore be possible to study the locations of different proteins in ribosomes by selecting a given fraction of the ribosome protein with these gentle procedures.

Modified DEAE analysis for RNA. It has been reported (Year Book 59) that phenol-prepared ribosomal RNA was reproducibly eluted from DEAE at a salt concentration of 0.8 *M* NaCl. The elution medium also contained 0.01 *M* tris buffer at pH 7.4, and Mg^{++} 10^{-2} *M*.

Present samples of DEAE did not give this simple elution pattern. Only 30 per cent of the ribosomal RNA was found to be eluted at 0.8 *M* NaCl using a linear salt

gradient of 0.02 to 1.3 *M*. A procedure was developed for increasing the yield of RNA from the column to 80 or 90 per cent of the amount put on.

By superimposing a pH and tris gradient on the NaCl gradient it was found that the ribonucleic acid only poorly eluted at 0.8 *M* NaCl was now eluted at salt concentrations between 0.65 and 0.75 *M* NaCl. The system used was:

(a) Low salt chamber: 0.02 *M* NaCl, 10^{-2} *M* Mg^{++} , 0.01 *M* tris pH 7.4.

(b) High salt chamber: 0.9 *M* NaCl, 10^{-2} *M* Mg^{++} , 0.05 *M* tris pH 8.8.

In this system ribosomal RNA eluted at 0.65 *M* NaCl, and 85 per cent of the RNA loaded was recovered. Unfortunately, S-RNA now eluted at 0.55 *M* NaCl and interfered with the front of the peak. Stretching the gradient obviated this interference.

Comment. These measurements cleared up some earlier confusion that had resulted from running the columns at 5×10^{-3} *M* magnesium. Richard B. Roberts.

IV. PROTEIN SYNTHESIS

A. Correlation with Ribosomes

IV.A.1 Synthesis of Nascent Protein by Ribosomes in Escherichia coli

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Introduction.—Cells and tissues of all kinds of living organisms have been found to contain ribonucleic acid (RNA) and protein both as separate constituents and as complexes in the form of ribonucleoprotein particles. From a variety of experimental data it has been inferred that these particles are probably intimately involved in the processes of protein synthesis.

The term microsome was originally applied to the portion of disrupted animal tissue which sedimented between 20,000 and 100,000 $\times g$.^{1, 2} It was found to contain most of the RNA. Later it was established that microsome preparations consisted of an association of ribonucleoprotein particles with fragments of the endoplasmic reticulum and techniques were evolved for removing the latter to yield particles which contained little else besides RNA and protein (see review by Palade³). Similar particles have also been isolated from bacteria⁴ and the term ribosome has been suggested for the purified particles which are essentially free from lipid and other extraneous matter.⁵

In bacteria, depending on the cultural conditions, up to 80 per cent of the RNA may exist in varying sized ribonucleoprotein particles. These range in sedimentation constants from about 20S to 100S and some preparations have about 2 amino acid residues per ribonucleotide. The distribution among the various classes found in *Escherichia coli* depends on the cultural conditions and the conditions of cell breakage. Exponentially growing organisms possess ribosomes with sedimentation constants approximately 15–20S, 30S, 50S, 70S, 85S, 100S. The last is usually a minor component or absent except in resting cells and the 70S and/or 85S particles predominate over the smaller sizes.^{6, 7} The sedimentation constant of the particle which we refer to as 85S does in fact vary between 70S and 100S depending on the magnesium concentration.⁸

Separation of the various particles into homogeneous fractions presents considerable difficulties if attempted by simple differential centrifugation. We have found, however, that centrifugation of cell juices layered on sucrose solutions in the swinging bucket rotor of the Spinco can give a useful resolution of all classes of ribosomes seen in the analytical ultracentrifuge. In addition the isolated fractions are available for analysis. This technique has been used to observe the incorporation of radioactive tracers into the ribosomes and the soluble protein of *E. coli*.

Methods.—The procedures described below were followed except for minor modifications. A radioactive tracer was added to an exponentially growing culture of *E. coli* and, after an appropriate interval, the organisms were fractionated to determine the distribution of the tracer among the various sizes of ribosomes and in the soluble protein.

E. coli B (ATCC No. 11303) was grown overnight at 37°C in an aerated synthetic medium containing glucose (0.4 mg/ml) as carbon source. The yield of organisms was approximately 120 μ g dry weight per ml of culture and in the morning more glucose (0.8 mg/ml) was added. This caused immediate resumption of exponential growth. The organisms were harvested after the culture density had about doubled. The cells were washed twice and suspended in a synthetic medium lacking both glucose and the sulfur source. Glucose (0.8 mg/ml) was added and the cells (suspension density of 250 μ g/ml) were incubated with aeration at 37°C. Exponential growth was promptly resumed at the expense of endogenous sulfur reserves.⁹

After 30 minutes the culture was poured into a beaker containing carrier-free $\text{Na}_2\text{S}^{35}\text{O}_4$ at a level of 1 mC per liter of culture. After a few seconds the whole suspension was dumped on to half its weight of crushed, frozen medium. This caused the temperature to drop precipitously and effectively halted metabolic activities instantly.

The organisms were then harvested, washed three times with TSM (tris(hydroxymethyl)aminomethane 0.01 *M*, succinic acid 0.004 *M*, MgAcetate 0.01 *M*, pH 7.6)

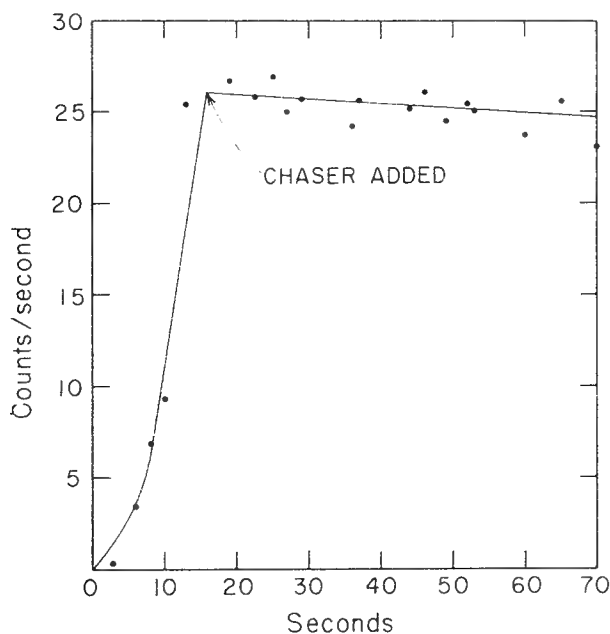


FIG. 1. —Time course of incorporation. $\text{S}^{35}\text{O}_4^-$ was added to a growing culture of sulfur-depleted *E. coli* at time 0. Samples were withdrawn at indicated times, and squirted into TCA, filtered and counted. At 16 seconds $\text{S}^{32}\text{O}_4 = \text{S}^{32}$ cystine and methionine were added. Note prompt incorporation of the tracer into TCA precipitable material and rapid cessation of incorporation after addition of nonradioactive material.

and resuspended in TSM (7.5 mg dry weight/ml). The cells were broken either by passing through an orifice under high pressure or by the lysozyme-freezing method.¹⁰ Residual cells, cell walls, and membranes were then removed by centrifugation. The resulting cell juice was then ready for sedimentation analysis as follows.

A mixing device¹¹ was used to deliver 4.5 ml of a solution of sucrose in TSM linearly graded from 20 per cent w/v to 5% w/v into a centrifuge tube. On this was layered 0.5 ml of a solution graded from 4 per cent to 0 per cent sucrose and containing the cell juice in reversed concentration gradient. This system has inherent stability properties which make it valuable for centrifugal separation of components of different sedimentation constants.¹² The tubes were spun in the swinging bucket head rotor (90,000 g) for periods varying from 75 to 200 minutes. Twenty-five fractions were collected from each tube by puncturing the base with a hypodermic needle and allowing the contents to drip out into small test tubes. The ultraviolet absorption of the fractions was measured. In addition, the radioactivity precipitable by trichloroacetic acid (TCA) was determined. All operations except the final collection of the fractions were carried out at 0–4°C.

Results.—As a preliminary to the study of the incorporation into ribosomes, we investigated the kinetics of $S^{35}O_4^-$ fixation into the total cold TCA precipitable fraction of *E. coli* and the rapidity with which this incorporation could be stopped by lowering the temperature or by adding a “chaser” containing non-radioactive SO_4^- , cystine and methionine. Samples were removed with a hypodermic syringe as rapidly as possible from a culture after addition of $S^{35}O_4^-$, squirted into TCA, filtered and counted.¹³ After an appropriate time the nonradioactive substrate

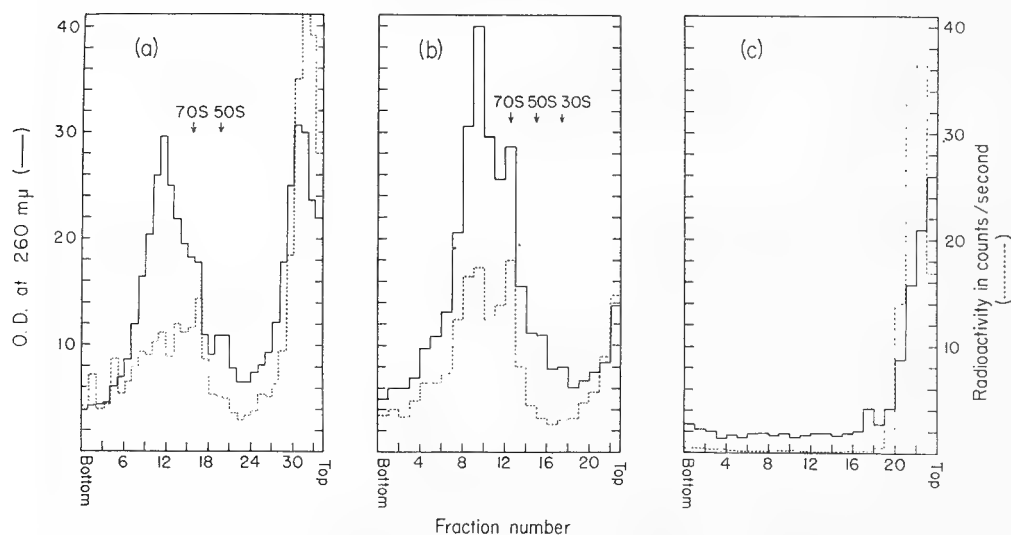


FIG. 2.—Sedimentation analysis of cell juice after 15 seconds incorporation of $S^{35}O_4^-$. $S^{35}O_4^-$ was added to a growing culture of sulfur-depleted *E. coli*. After 15 seconds the culture was chilled, harvested, washed, and broken. The cell walls and membranes were spun out and the juice analyzed in the swinging bucket rotor; 2a shows analysis of the total juice; 2b analysis of resuspended ribosome pellet; 2c analysis of supernatant fluid after removal of ribosomes. The 85S, 70S, 50S, and 30S particles seen in the analytical centrifuge are partially resolved. Note lack of contamination of ribosome region by soluble protein, 2c. Centrifugation 75 minutes at 37,000 rpm.

was added and the sampling continued. As shown in Figure 1 a linear rate of incorporation was found after a few seconds and this could be obliterated instantly when the “chaser” was added. These experiments were carried out with cells which had been grown for about 30 minutes without an exogenous source of sulfur.

The absence of kinetic delays in the incorporation of S^{35} and in its cessation indicate that under these conditions of sulfur starvation there is little if any pool of precursors to the sulfur amino acids of the cell protein.

In other experiments the culture was poured on ice a few seconds after adding the tracer. Samples taken from the iced suspension during the following 2 hours showed a rate of incorporation less than $1/5000$ of the rate at 37° . Furthermore, none of the S^{35} which had been previously incorporated into TCA precipitable material was removed by exchange in the presence of chaser at the low temperature. Accordingly, changes in the content of TCA-precipitable S^{35} occurred during the period when the cells were incubated at 37° and not during the period required for harvesting, washing, and fractionating the cells, all of which were carried out between $0-4^\circ\text{C}$.

Experiments of a few minutes' duration had consistently failed to show a higher specific radioactivity in the ribosomes than in the soluble protein. We, therefore, investigated the distribution of S^{35} after very short times of incorporation. Organisms were incubated for 10 seconds with $S^{35}\text{O}_4^{2-}$ and their components were separated by sedimentation analysis. The distribution of TCA-precipitable radioactivity and 260 $m\mu$ absorbing material is shown in Figure 2a. Most of the S^{35} is seen to be associated with the ribosomes of the 70–85S class and with the soluble protein which did not sediment under the conditions used. When the ribosomes were pelleted from such a juice and the resuspended pellet similarly analyzed, most of the soluble protein fraction was eliminated but the 70S particles still carried their

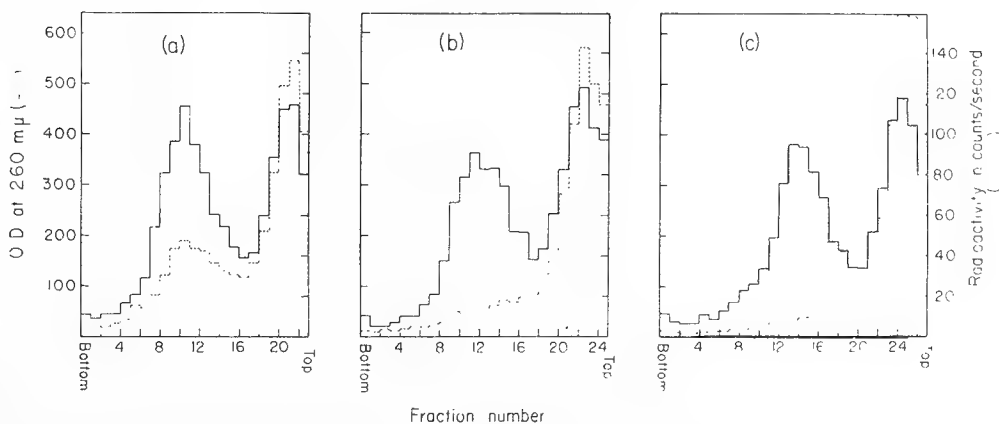


FIG. 3.—Sedimentation analysis of cell juice. (A) Cells incubated 15 seconds with $S^{35}\text{O}_4^{2-}$. (B) Cells incubated 15 seconds with $S^{35}\text{O}_4^{2-}$ followed by 15 seconds incubation with S^{32} "chaser." (C) Fifteen seconds incubation with $S^{35}\text{O}_4^{2-}$ followed by 120 seconds with S^{32} "chaser." Note transfer of radioactivity from 70–85S region to nonsedimenting region. Centrifugation 75 minutes at 37,000 rpm.

original radioactivity (Fig. 2b). This indicates that the S^{35} was relatively firmly bound to the ribosomes (see also below). When the supernatant fluid remaining after the ribosomes had been pelleted was analyzed, very little of the radioactivity moved down the centrifuge tube (Fig. 2c).

In a variety of experiments of short duration (5–20 seconds) the major part of the label associated with ribosomes was found in the 70–85S fraction and the specific

radioactivity of these particles was always substantially greater than that of the 30 and 50S ribosomes. When incubations lasting 20, 45, and 120 seconds were used and the resulting cell juices analyzed, it was found that the specific radioactivity of the 70S particles had risen rapidly by 20 seconds and thereafter increased only a little up to 120 seconds. The specific radioactivity of the 50S ribosomes rose more slowly but surpassed that of the larger particles between 45 and 120 seconds. The soluble protein also became labeled rapidly but because of the much greater amount had lower specific radioactivity at the earlier time.

To demonstrate that one component of the cell acts as precursor to another it is necessary to show not only that the radioactivity of the suspected precursor rises rapidly when a tracer substrate is added but also that it is transferred to the product with equal rapidity when the radioactive substrate is replaced by a nonradioactive one. Cells were therefore incubated with the radioactive tracer for 10–15 seconds and then a “chaser” of nonradioactive amino acids was added. After a short period all incorporation was terminated by pouring the culture on ice. Figure 3 shows that most of the radioactivity which was found in the ribosome fraction at the end of 15 seconds was subsequently transferred to the soluble protein during the 15 seconds of incubation with the “chaser.” The removal was only slightly greater in 120 seconds. Even a 5 second “chase” was quite effective. Thus there is a protein component which is transiently associated with the ribosomes and has all

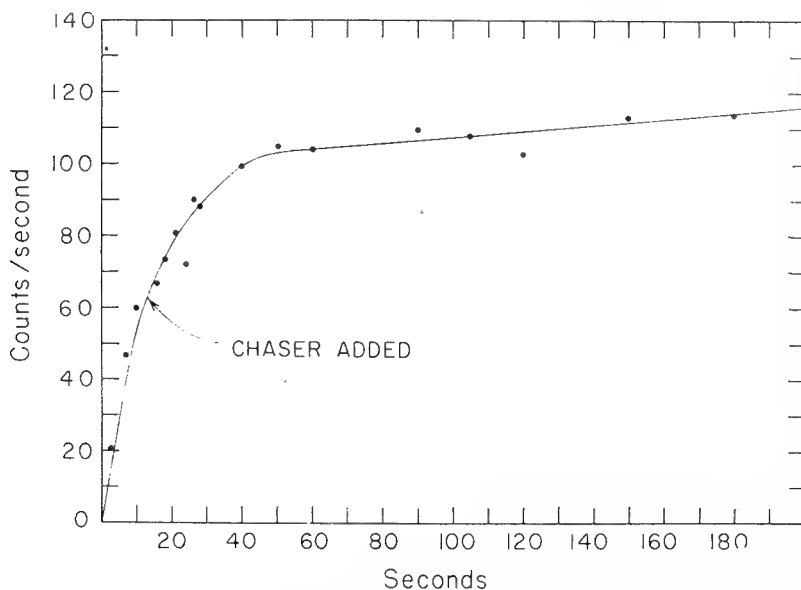


FIG. 4.—Time course of incorporation of C^{14} amino acids. Experimental conditions similar to those of Fig. 1 except C^{14} labeled *Chlorella* protein hydrolysate was used as tracer. Note that incorporation continues after addition of C^{12} amino acids.

the characteristics which would be expected in a compulsory precursor of the soluble proteins. It appears that this nascent protein is a polypeptide strand which is formed on the ribosome and is subsequently released as soluble protein. Owing to the short time periods involved it has not been possible to plot detailed

time courses of these processes but three features have been established. (1) The radioactivity of the 70–85S ribosomes built up to a saturation level in 5 seconds or less and died away equally rapidly when the tracer was diluted out. (2) The saturation level was equivalent to the quantity of soluble protein synthesized in three seconds. (3) The decrease in the radioactivity of the ribosomes during the chase was roughly equal to the concomitant increase of radioactivity in the soluble protein.

To check whether the results obtained with S^{35} incorporation were typical of other amino acids, similar experiments were carried out to observe the incorporation of a mixture of amino acids.† Figure 4 shows that the incorporation of C^{14} from the mixture of C^{14} amino acids (obtained by hydrolyzing *Chlorella* protein) began promptly but could not be terminated rapidly by adding a large excess of C^{12} amino acids. Exchange of exogenous amino acids with the amino acids of the pool is not very rapid and C^{14} continued to enter the protein fraction for roughly 20 seconds after the “chaser” was added. Consequently a longer period was needed to show the transfer of the particle-bound nascent protein to the soluble fraction. The results, however, were quite similar to those obtained with S^{35} . After 5 and 12 seconds a large fraction of the C^{14} incorporated into the TCA-precipitable fraction was associated with the 70–85S particles as shown by the sedimentation analysis. During the subsequent 120 seconds after the addition of C^{12} amino acids the C^{14} was transferred

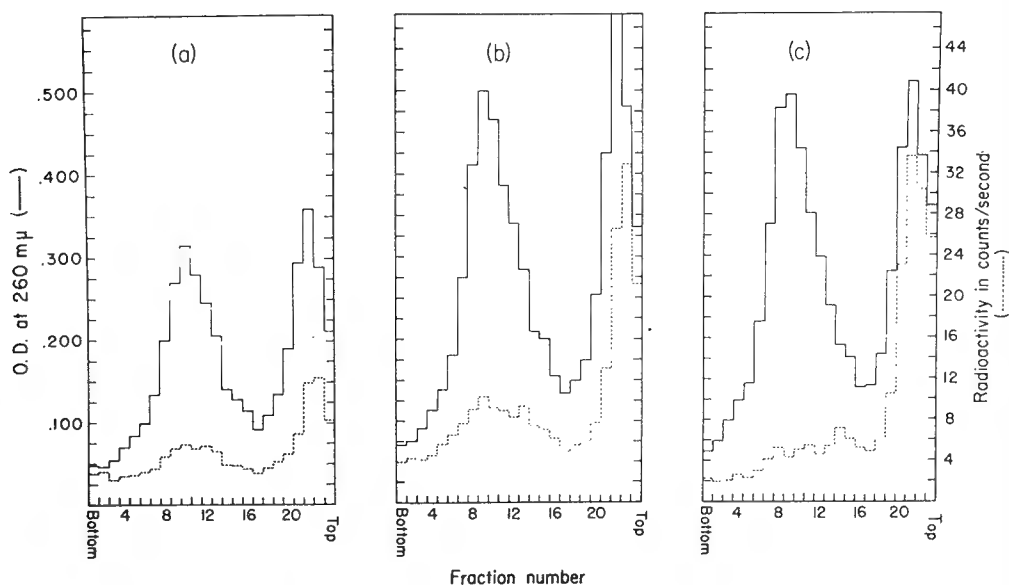


FIG. 5.—Sedimentation analysis of cell juice. (A) Cells incubated 5 seconds with C^{14} amino acids. (B) Twelve seconds incubation. (C) Twelve seconds incubation with C^{14} amino acids followed by 120 seconds incubation with C^{12} amino acids. Note decrease in radioactivity of 70–85S region and increase of radioactivity in nonsedimentable region. Centrifugation 75 minutes at 37,000 rpm.

to the soluble fraction (Fig. 5). As a result of the longer times required to dilute out the free amino acid pools there was more opportunity for incorporation into the structural proteins of the ribosomes and the transfer was not quite so complete as could be observed when using the S^{35} tracer. Neither was it possible to demon-

strate the rapidity of the transfer from the particle to the soluble fraction. Nevertheless the results show that the behavior of the S^{35} amino acids is consistent with that of the other amino acids and can be used with confidence to study the processes of protein synthesis.

In the experiments described above we have taken TCA-precipitability as an indication that the labeled amino acids were in peptide linkages. There exist, however, complexes of soluble RNA or lipids with amino acids which are precipitated by cold TCA. A number of tests were therefore carried out to establish the nature of the labeled material which is transiently associated with the ribosomes. (1) It remained precipitable by TCA after solution in 1 *N* NaOH. (2) It was not extractable by hot alcohol after cold TCA precipitation. (3) It was not exchangeable by incubation with an excess of nonradioactive amino acids. (4) It yielded a large variety of compounds (peptides) having different electrophoretic mobilities after partial hydrolysis by chymotrypsin or 12 *N* HCl. These tests indicate that the bulk of the newly incorporated amino acids which were found associated with the ribosomes have the behavior to be expected of amino acids bound in the peptide linkage.

The association of the nascent protein with the ribosomes, although transient in the growing cell, is quite stable in the disrupted cell juice. Preparations have shown the same specific activity in the ribosome fraction after 5 days at 4°C.

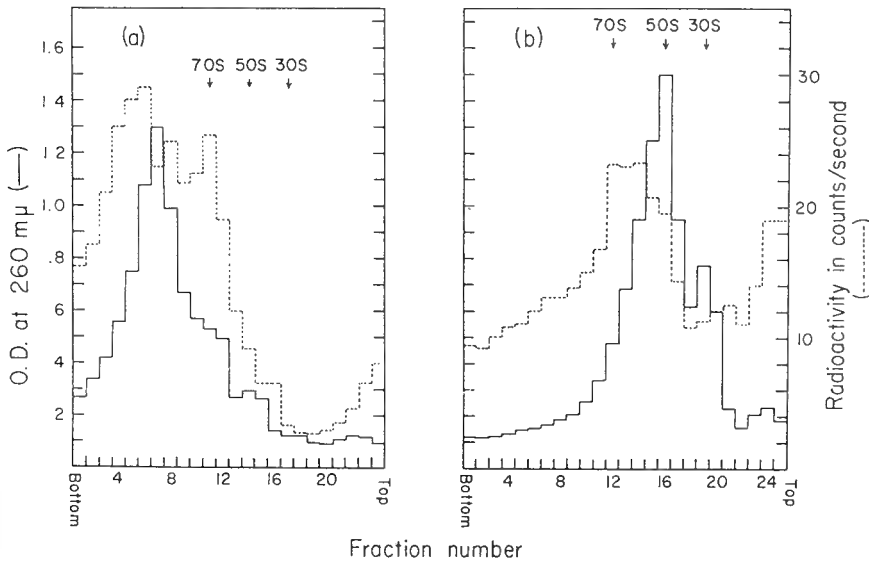


Fig. 6.—Sedimentation analysis of ribosomes from cells exposed for 10 seconds to $S^{35}O_4^{2-}$. (A) Ribosome pellet resuspended with buffer containing Mg at 10^{-2} M to give mostly 70–85S ribosomes. (B) Pellet resuspended with Mg at 10^{-4} M to give mostly 50S and 30S ribosomes.

The ribosomes may be centrifuged down from the cell juice and the pellet shows the same specific radioactivity in the 70S region upon subsequent sedimentation analysis. We have not yet found conditions which cause the release of the nascent protein from the ribosomes. Incubation (15 minutes at 37°) with adenosine triphosphate (ATP) or with amino acids in the presence of cell juice did not release it.

Chromatography with diethylaminoethyl (DEAE) cellulose caused disintegration¹⁴ of the ribosomes yielding nucleoprotein which was eluted from the column but leaving about one-half of the ribosome protein and 95 per cent of nascent protein firmly bound. On splitting the ribosomes by decreasing the magnesium concentration of the medium,¹⁵ the nascent protein was found partly in the soluble proteins but mainly in the 50 and 30S particles which comprised the bulk of the nucleoprotein and in a few remaining 70S ribosomes (Fig. 6).

On the other hand, the soluble protein which was labeled with S^{35} as early as 7 seconds after addition of the tracer could not be distinguished from the bulk of the unlabeled soluble protein by chromatography on DEAE. There is no indication from column chromatography that any appreciable time is required (in growing cells) for conversion from nascent protein associated with the ribosomes to the final form of the polypeptide chain.

A less detailed study was made of the incorporation of S^{35} in the fraction of broken cells which sedimented rapidly. This contained fragments of cell walls and membranes as well as some intact cells. Under steady state conditions of labeling roughly 25 per cent of the total S^{35} was in this fraction whereas organisms which had been incubated with tracer for only 10–15 seconds showed about 30 per cent. This might be taken to indicate the presence of a protein precursor. However, sedimentation analysis indicated that half of this material sedimented very rapidly ($>1000S$) as might be expected for cell walls and unbroken cells. A large part of the remainder is accounted for by contamination with ribosomes and soluble protein leaving only a minor component which has the sedimentation properties to be expected of small fragments of cell membrane. The specific radioactivity of this fraction was similar to that of the soluble protein. Furthermore, its radioactivity did not diminish after the addition of a "chaser." Thus, there is no evidence that this fraction contains a major protein precursor. There is, however, a slight indication that ribosomes associated with cell membranes may be more active in protein synthesis. When cells were lysed by treatment with lysozyme followed by freezing and thawing about half of the ribosomes were released and most of the remainder were detached from the residual membranous material by passing it through the pressure cell. It was found that the first fraction of ribosomes had only about half the specific radioactivity of the second. Possibly some of the particles exist free in the cell juice whereas others are more or less firmly bound to membranes and are more directly involved in protein synthesis.

Discussion.—Various lines of evidence suggest that the microsome system in animal cells may be the predominant site of protein synthesis. For instance, amino acid incorporation studies *in vivo* show that this cell fraction becomes labeled most rapidly and only subsequently does the soluble protein fraction become radioactive.^{16,17} Similar results have been obtained with pea seedling preparations.¹⁸ Support for these ideas has come from *in vitro* experiments in which suitably supplemented microsome preparations have been shown to incorporate amino acids into peptide linkage.¹⁹ However, the incorporation was relatively small and boiled preparations were reported to be substantially more active.²⁰

In contradistinction, studies with bacterial cell preparations had not hitherto indicated that ribosomes were an important site of protein synthesis. This is surprising in view of the fact that bacteria have a considerably higher content of

ribosomes than do animal cells. Some recent reports, in fact, propose that the bacterial cell membrane is a relatively more important structure in such synthesis.²¹⁻²³

In animal tissues, cellular proliferation is small compared with synthesis of soluble protein so there is little difficulty in distinguishing between synthesis of and by ribosomes. In certain cells there is no synthesis of ribonucleoprotein particles so that all incorporation of amino acids can be attributed to synthesis of soluble protein by the ribosomes.²⁴ In contrast, in growing cultures of bacteria the ribosome content will double in a mean generation time as will the amount of soluble protein.

E. coli in the exponential phase of growth in a synthetic medium has a mean generation time of about 50 minutes and contains approximately 40 per cent soluble protein, 10 per cent ribosomal protein, and 10 per cent cell wall protein, all expressed on a dry weight basis. In a single cell there are roughly 6000 of the 70-85S ribosomes and 6×10^6 arbitrary units of 10,000 MW in the soluble protein. As the growth rate is 0.02%/second, 1.2 ribosomes and 1200 protein units will be synthesized per second. Assuming that the ribosomes are the site of protein synthesis there may be as many as 6,000 sites operating. If this were so, the average time required for the completion of a protein unit would be five seconds. If these nascent protein molecules leave the ribosomes as soon as they are completed, then only those protein molecules synthesized during the previous five seconds will be found adhering to the ribosomes.

At the same time amino acids are flowing in to form the structural proteins of the ribosomes at one-quarter the rate that amino acids are required for synthesis of soluble protein. Hence after twenty seconds the total quantity of new ribosomal protein will equal the quantity of adhering nascent protein. Earlier experiments¹⁴ failed to detect the presence of the nascent protein because periods as long as four minutes were allowed for incorporation. At the end of four minutes the nascent protein would be only 10 per cent of the newly formed ribosome protein.

Fortunately, techniques are available to recognize these two types of synthesis. A 5-15 second "chase" will displace the nascent protein but does not affect the newly formed ribosome protein which is a permanent end product. In other studies carried out in this laboratory^{25, 26} it was found that longer incubations (1-30 minutes) with $S^{35}O_4^{=}$ followed by a short "chase" gave a pattern of labeling which implied that the smaller ribosomes were made first and were themselves precursors to the larger, 30 and 50S particles combining to yield 70 and 85S particles. Accordingly, the two types of synthesis are partially separated according to particle size, the larger particles carrying most if not all of the nascent protein whereas amino acids newly incorporated into the structure of the ribosomes appear at first in the smaller particles. Furthermore, the use of $S^{35}O_4^{=}$ as a tracer emphasizes the nascent soluble protein as the sulfur amino acid content of the ribosomal protein is one-half to one-third lower than that of the soluble protein. As a result, the radioactivity appearing in the 70-85S particles after incubation with $S^{35}O_4^{=}$ for 15 seconds or less is chiefly due to nascent protein and there is little contamination from protein of newly formed ribosomes.

The results reported here indicate that a time scale of 5 seconds for the completion of protein units is approximately correct. Free amino acid pools might introduce some slight kinetic delays which would increase the apparent time but there is no doubt that the nascent protein has a transient existence of less than 5-10 seconds in the growing cell.

It is not possible from these experiments to determine the size of the polypeptide strands which make up the nascent protein as this calculation requires a knowledge of the fraction of the ribosomes that is active. The 30 and 50S ribosomes are certainly not the major loci of the nascent protein but they are only minor components and it cannot be excluded that they carry a correspondingly small part of the nascent protein. Their role in forming nascent protein is obscured by the rapid labeling of their structural protein.

It is possible that both the 70 and 85S particles synthesize nascent protein but the former consistently show an initial higher specific radioactivity. Hence, it seems more likely that the nascent protein is formed on the 70S particles and is carried on to the 85S by a rapid interchange between these forms. Finally, it is also possible that only the 70S particles associated with the cell membrane are active. If we assume, however, that the 70S particles are all equally active and that the other particles are inert, and if we take 5 seconds as the time for formation of a polypeptide strand, then the product must have a molecular weight of roughly 20,000.

At first sight it might appear that a growing culture of bacteria is a system so complicated as to be less useful in a study of the problems of protein synthesis than are more simplified systems. It has become apparent, however, that by choosing appropriate time scales one can emphasize one or another aspect of the total cell synthesis. When this is done it is possible to show both the rapid build-up and decay of radioactivity in the nascent protein attached to the ribosomes and to show its transfer to the soluble protein. Furthermore, it is possible to show that this process occurs in the appropriate quantity to account for most if not all of the synthesis of the soluble protein. Finally, it is possible to study separately the synthesis of protein by ribosomes and the synthesis of the ribosomes themselves even though they are intimately coupled in the intact growing cell.

Summary.—The ribosomes of *Escherichia coli* can be separated into different sedimentation classes by centrifugation of cell juices through solutions of sucrose.

Radioactivity from $S^{35}O_4^{2-}$ and C^{14} -amino acids rapidly appears in protein bound to the 70S and 85S ribosomes. These become saturated within a few seconds and equally rapidly shed this nascent material as soluble protein which cannot be distinguished from the main bulk of soluble protein.

The rate of formation of this nascent protein on the larger ribosomes is adequate to account for the total cell protein synthesis.

This process is distinct from the concomitant synthesis of the proteins which constitute the permanent structure of the ribosomes.

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† These experiments were carried out with Dr. R. Hendler who used a portion of the cultures to measure the kinetic behavior of lipid-bound amino acids.

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Comment. Although we did not realize it at the time, the data of this paper provide support for the polysome theory (J. R. Warner, P. M. Knopf, and A. Rich, *Proc. Natl. Acad. Sci. U. S.*, **49**, 122-129, 1963; H. M. Goodman and A. Rich, *Nature*, **199**, 318-322, 1963). The heavy fractions contained an excess of nascent protein, particularly when gentle methods were used to break the cells. The quantity of nascent protein corresponds more closely to one strand per ribosome than to one strand per template. The nascent protein is more firmly bound to the ribosomes than nascent RNA is. The data of figure 6 have been interpreted to mean that only a small fraction of the ribosomes are engaged in protein synthesis. The correct interpretation is probably that only a small fraction of the ribosomes carry sufficient nascent protein to protect them from disintegration into smaller particles. Richard B. Roberts.

IV.A.2 Ribosome-Bound β -Galactosidase

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Considerable evidence has been accumulated to show that ribonucleoprotein particles (ribosomes) provide sites for protein synthesis in animal cells.¹ Studies of the incorporation of radioactive tracers by *Escherichia coli* also indicated that the ribosomes of bacteria are active in protein synthesis (McQuillen, Roberts, and Britten²). These authors showed that in growing bacteria, a quantity of material roughly equal to the protein synthesized in three seconds was transiently associated with the ribosomes before being released to the soluble protein fraction of the cell. Since the rate of protein synthesis in *E. coli* is about 0.02 per cent per second, 0.06 per cent of any *particular* protein might be expected to be found transiently associated with the ribosomes. A series of experiments was started to determine whether β -galactosidase showed the same transient association with ribosomes as was indicated for proteins in general. The first experiments showed that a small fraction of the enzyme was bound to the ribosomes, and it is the purpose of the present paper to describe some of the properties of this ribosome-associated enzyme. Furthermore, the results suggest a general procedure for isolating specific ribosomes.

Materials and Methods.—E. coli strains: Three strains of *E. coli* differing in their β -galactosidase-synthesizing properties were used, namely ML 30 (inducible), ML 308 (constitutive), and W2214 (absolute negative).

Growth conditions: Cells were grown at 37°C in a vigorously aerated synthetic medium (C) of the following composition: 2 g NH₄Cl, 6 g Na₂HPO₄, 3 g KH₂PO₄, 3 g NaCl, 0.01 g Mg as MgCl₂, 0.026 g S as Na₂SO₄, 900 ml H₂O, and 10 ml. of 10 per cent maltose.

Enzyme induction and assay: Thiomethyl- β -D-galactoside (TMG) or thioisopropyl- β -D-galactoside (TIPG) at 5×10^{-4} M were used as inducers of β -galactosidase synthesis. Assays of β -galactosidase on ribosomal preparations were performed with the following mixture buffered at pH 7.4; 0.0027 M ortho-nitrophenyl- β -D-galactoside (ONPG), 0.05 M NaCl, 0.01 M trishydroxymethylaminomethane

(Tris), 0.004 M succinic acid, and 0.01 M magnesium acetate. The hydrolysis of the ONPG was followed in a Beckman spectrophotometer at 420 mμ. The sodium chloride, tris-succinate mixture was used in place of the more commonly employed phosphate buffer because of the known³ instability of ribosomes in the presence of phosphate. The rate of ONPG hydrolysis is the same in the two buffer systems.

Preparation of wall-free cell juice: Exponentially growing cultures of *E. coli* were harvested and washed once in a Tris buffer adjusted to pH 7.6 containing 0.01 M Tris, 0.004 M succinic acid, and 0.01 M magnesium acetate (TSM). Following the wash, the cells were resuspended in 10 ml of the same buffer. The cells

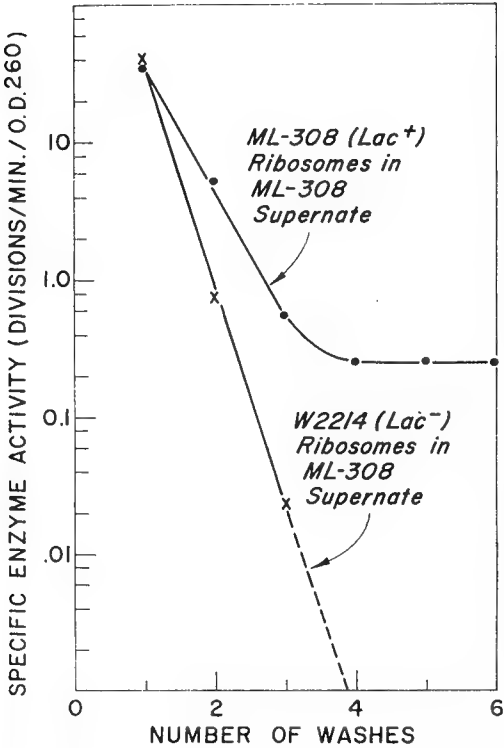


FIG. 1.—The effect of successive washings by centrifugation on the specific enzyme activity of ribosomes from genetic positives (●) and ribosomes from genetic negatives (X), both initially suspended in an extract containing large amounts of active β-galactosidase.

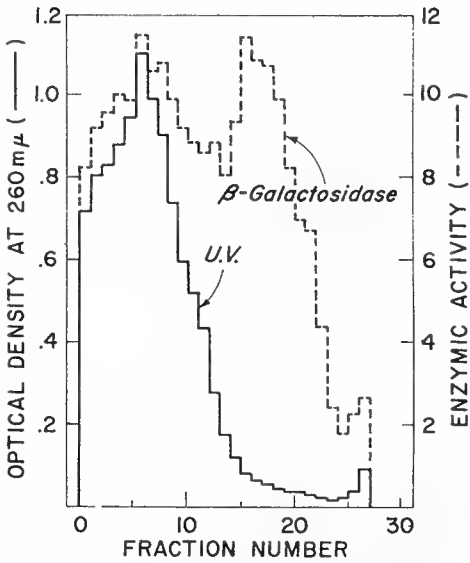


FIG. 2.—Sedimentation pattern of Lac⁺ ribosomes washed three times prior to being layered on the sucrose gradient (3 to 20 per cent). The run was made at 37K for 80 min.

of this suspension were ruptured by extrusion through a small orifice under pressure (approximately 15,000 lb/sq. inch) in a modified French pressure cell. The extruded material was centrifuged for five minutes at 40,000 rpm in the angle head rotor of the Spinco Model L centrifuge to remove whole cells, cell walls, and other large fragments.

Antisera: β-galactosidase antiserum was prepared by injecting rabbits with 10 mg of purified (90 per cent) *E. coli* β-galactosidase. Chicken anti-rabbit serum was kindly furnished us by Dr. Alan Boyden, Rutgers University, New Brunswick, N. J.

Results.—Enzymatic activity of ribosomes: Centrifugation of a wall-free cell

juice for 45 minutes at 40,000 rpm in the angle head rotor of the Spinco Model L centrifuge gives a pellet (40K 45P) which contains more than 90 per cent of the 70S and 85S ribosomes of the cell. Such a centrifugation leads to a useful separation of the major ribosome components from the bulk of the soluble proteins and smaller particles and yields material suitable for further purification.

One technique for purifying large quantities of 85S and 70S ribosomes involves repeated washing in TSM. Figure 1 compares the amounts of enzymic activity found in the pellet fractions (40K 45P) in successive washings of ribosomes derived from ML 308, the constitutive mutant (circles). It will be noted that by the fourth washing a constant specific enzymic activity (enzyme units per O.D. at 260 m μ) is achieved.

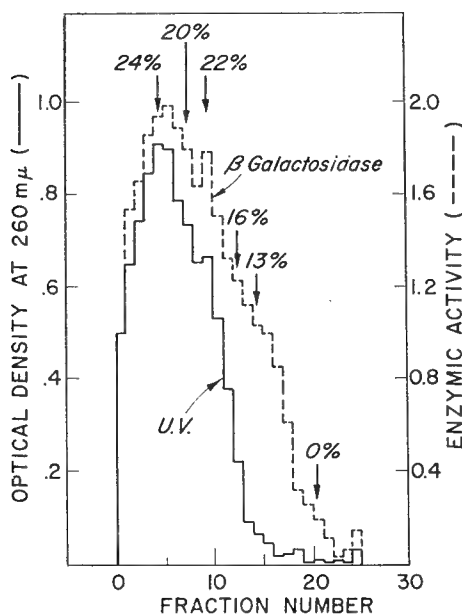


FIG. 3.—Sedimentation pattern of the ribosomes in the first 13 fractions of Figure 2. The numbers denote per cent increases in enzyme activity when the corresponding fraction was incubated with anti- β -galactosidase serum.

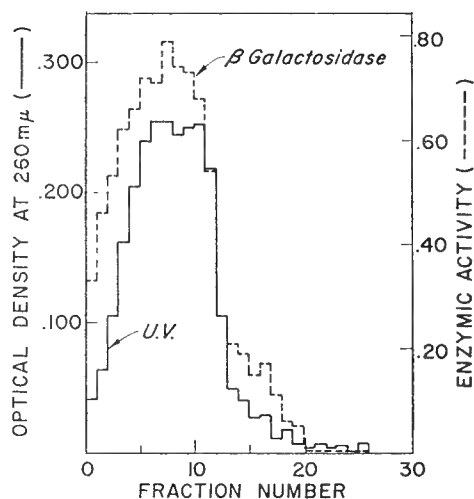


FIG. 4.—Sedimentation pattern of the ribosomes in the first 13 fractions of Figure 3.

Such data do not necessarily establish a specific association between a fraction of the enzyme molecules and the ribosomes. For example, some enzyme molecules could be nonspecifically but firmly adsorbed to the surface of the ribosomes. Alternatively, the enzymic activity of the pellet may not be associated with the ribosomes but may reside in aggregates possessing an average sedimentation constant roughly equivalent to the larger ribosomes.

The first of these possibilities was excluded by the following experiment. An extract rich in enzyme but free of ribosomes was prepared from the constitutive strain, ML 308 (Lac⁺), by centrifuging the cell juice for five hours at 40,000 rpm. Purified ribosomes prepared from the absolute lactose-negative strain W2214 (Lac⁻) were suspended in this extract. The resulting mixture was subjected to the

successive centrifugations in TSM. Figure 1 shows that the enzyme associated with the genetically negative ribosomal pellet behaves quite differently from that observed with genetically competent material. Initially, the specific enzymic activities of the two ribosomal pellets are about equal. In the lower curve, there is no suggestion of an approach to a constant specific activity on repeated washings. By the fourth step, the Lac⁻ ribosomes reach a specific activity (not indicated on the graph) which is less than 0.001 units of enzyme per optical density unit. Such enzymic activities are too low for accurate measurement and correspond to levels considerably less than 1/100 of the constant specific activities attained with the Lac⁺ ribosomes.

Centrifugation of a layer of ribosome suspension through a sucrose gradient in the swinging bucket rotor of the Spinco centrifuge⁴ provides a much better indication that the enzyme is actually associated with the ribosomes.

Figure 2 shows the distribution of the enzymic activity and optical density of a ribosome preparation (subjected first to two washes as described above) after being layered on the sucrose gradient and centrifuged for 45 minutes at 37,000 rpm. The enzyme is distributed roughly equally between the ribosomal and supernatant fractions. Figures 3 and 4 illustrate how successive sedimentations through sucrose gradients lead to the elimination of the soluble enzyme component from the ribosome fraction. The first 13 fractions of the run described in Figure 2 were pooled and the ribosomes collected by centrifugation (40K 45P). They were then resuspended and layered on a new sucrose gradient and centrifuged. The resulting profile of O.D. at 260 m μ and of enzyme activity is given in Figure 3. Comparatively little enzyme is found unassociated with the ribosome peak although there is clearly a contamination of free enzyme. A repetition of this procedure on the first 13 fractions of Figure 3 is shown in Figure 4. Here, there is an excellent correlation of enzyme activity with ribosome peaks in the density gradient.

Nature of the ribosome-associated enzyme: The sedimentation patterns described above encouraged the belief that some enzyme molecules are in physical association with the larger ribosome particles. It was of interest to look for some feature other than the sedimentation characteristics which would serve to distinguish these molecules from those which are in the soluble fraction. A series of experiments were, therefore, performed examining the effect of a variety of agents on the residual ribosomal enzyme activity. Methods of disrupting ribosomes (RNAase, versene, or citrate) which have been shown to be capable of releasing latent RNAase activity of the ribosomes of *E. coli*^{5, 6} did not cause any increase in the β -galactosidase activity of highly purified ribosomal preparations. Several attempts to activate such preparations by the addition of the galactoside inducers, TMG and TIPG, were also unsuccessful.

The effect of adding specific antiserum was examined with the hope that it might serve as a specific means for removing the associated enzyme from the ribosomes. Table 1 shows that exposure of purified ribosomes to anti- β -galactosidase results in a striking increase in enzymic activity. In a series of similar experiments using comparably purified ribosomes, increases of between four- and sixfold were often observed. This activation is unique for the specific anti- β -galactosidase serum: normal rabbit serum and nonhomologous antisera (e.g. anti-alkaline phosphatase) have no effect. Further, the increase in activity is confined to ribosome-bound

TABLE 1

EFFECT OF RABBIT SERUM UPON RIBOSOME ASSOCIATED AND SOLUBLE β -GALACTOSIDASE

Fraction	Rabbit serum added	Enzyme activity
Ribosomes (uninduced ML 30)	0	0.04
Ribosomes (" ")	Anti- β -galactosidase	0.15
Ribosomes (" ")	Normal	0.05
Soluble (" ")	0	0.092
Soluble (" ")	Anti- β -galactosidase	0.090

Ribosome fractions were purified by successive centrifugations to constant specific activity and enzyme was assayed with ONPG according to the procedures described under *Methods*. Soluble enzyme was obtained by removal of ribosomes by means of a sucrose gradient swinging bucket centrifugation. Assays were continued until linear rates were well established.

enzyme and is not observed with the soluble enzyme as shown in Figure 3. Aliquots taken from fractions corresponding to different portions of this O.D. profile were tested for their ability to be activated by the specific β -galactosidase antiserum. The increases over controls are recorded in Figure 3 over the appropriate parts of the profile.‡ Fractions corresponding to the ribosome region exhibit the antiserum-activating effect. As one proceeds to fractions closer to the free soluble region, however, the degree of activation falls and finally becomes zero. Thus, the soluble enzyme and the enzyme associated with the ribosomes differ not only in their sedimentation rates but also in their response to antibody.

Two other possible explanations for such activation are (1) that the addition of ribosomes activates in some manner β -galactosidase present in the antiserum which is not detectable in their absence or (2) that the presence of ribosomes nonspecifically inhibits the activity of β -galactosidase and this inhibition can be reversed with specific antiserum. To test the validity of the first suggestion, purified ribosomes prepared from the Lac⁻ strain (W2214) were incubated with the anti- β -galactosidase serum. These mixtures were assayed for enzymic activity and none was found. It is concluded from such experiments that the observed increase in activity is not due to a latent enzyme in the antiserum. To examine the second possibility, the following experiment was performed. A soluble enzyme fraction was prepared from fully induced cells by exhaustive centrifugation of the cell-free extract to remove the ribosomal components. Purified ribosomes from a Lac⁻ strain of *E. coli* (W2214) were introduced into this soluble enzyme extract. The ribosomes were then purified in the usual fashion by successive centrifugations. The purification was stopped when the specific enzymic activity corresponded to that at which pronounced activation by antiserum is observed with Lac⁺ ribosomes (see Table 1). The resulting mixture was then exposed to antiserum and the enzyme activity measured. No activation of the enzyme was ever observed. In a typical experiment, 0.092 div./min were observed in the absence of antiserum and 0.090 div./min in its presence. Since the Lac⁻ ribosomes did not activate or depress the soluble enzyme activity, one is inclined to believe that the phenomenon is unique for ribosome-associated enzyme.

Mechanism of antibody action: Antibody was added to the ribosome preparations with the hope that the antibody might dislodge the enzyme molecule from the ribosome and thereby expose the active regions. A second possibility was that the antibody might help to shape the associated polypeptide without removing it from the ribosome. The sedimentation characteristics of the enzyme-antibody complex were therefore examined.

If the antiserum removes the enzyme from the ribosomes, one should no longer observe a peak of enzymic activity in the ribosome region. This possibility was tested under various conditions differing widely with respect to the amount of soluble β -galactosidase present. In one case, a soluble protein fraction from the constitutive mutant, containing a small quantity of ribosomes, was incubated with antiserum at room temperature for several hours. The amount of antiserum added was greatly in excess of that required to precipitate all the enzyme present. The mixture was then layered on a sucrose gradient and centrifuged at 37K for 90 minutes. A companion tube was run with enough purified ribosomes to show the position of the ribosome peak by its O.D. at 260 $m\mu$. Figure 5 shows the distribution of enzymic activity observed (dashed line) and of the O.D. at 280 $m\mu$ (light line) indicative of the protein distribution. Superimposed is the distribution of O.D. at 260 (heavy line) obtained in the companion tube. More than 99 per cent of the enzyme activity was precipitated as a pellet, and virtually none was found in the region corresponding to the soluble protein. There is, however, still a peak of enzyme activity in the region corresponding to the ribosomes.

The response of the ribosome-enzyme complex to antiserum was also examined using ribosome preparations at various stages of purification. In all cases, the same pattern was observed. Any soluble enzyme present was removed as a pellet, but there remained a residual peak of enzyme activity in the sedimentation pattern regions where the 70S and 85S ribosomes are located.

It is evident that antibody easily forms large precipitable aggregates with soluble enzyme but not with the ribosomal enzyme. Further, the activation effect of anti-serum does not seem to be produced by a removal of the enzyme molecules from their association with ribosomes. Were this the case, the enzyme either would precipitate rapidly, as does the soluble enzyme-antibody complex, or would show a decreased sedimentation constant if it were detached but not able to form an aggregate.

The association of the antibody with the ribosomes was demonstrated by the use of another antibody against the rabbit γ -globulin. A purified ribosome preparation was incubated for one hour in the presence of excess rabbit anti- β -galactosidase serum and then centrifuged for one minute at 25K. As expected, no detectable enzyme was removed from the supernatant, enzyme activities of the supernatants being 4.2 before and 4.7 per 0.1 ml after the centrifugation. In a control incubation and centrifugation with soluble enzyme, virtually all enzymic activity was removed

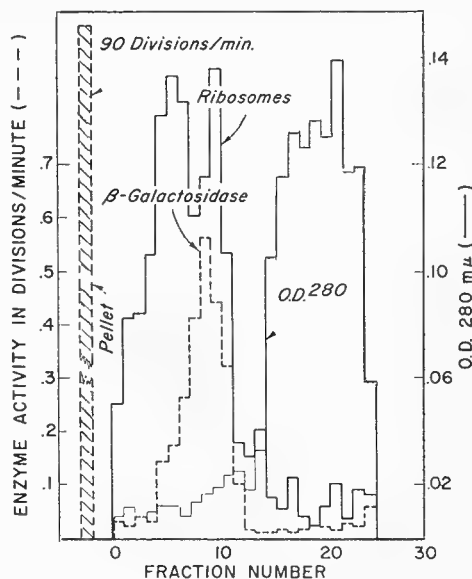


FIG. 5.—The effect of anti- β -galactosidase serum on the distribution of enzyme activity in a sucrose gradient sedimentation pattern of a small amount of ribosomes in the presence of excess enzyme. A companion tube was run to provide the position of the ribosomes as given by heavy line histogram.

from the supernatant. Subsequently, excess chicken anti-rabbit serum was added to the mixture of ribosomes and anti- β -galactosidase rabbit serum. After an hour's incubation, this mixture was subjected to a centrifugation (25K for 1 minute). Examination of the supernatant revealed that 94 per cent of the ribosome-bound enzyme had been precipitated. The enzyme removed from the supernatant was found in the pellet fraction. Examination of the ribosome content of the supernatant revealed that less than one per cent had been removed. Hence, less than one per cent of the ribosomes possess the β -galactosidase enzyme.

The amount of ribosome-associated enzyme in different states of induction: It was of interest to examine the effect of induction on the amount of β -galactosidase found associated with the ribosome fraction. Table 2 summarizes the results of a

TABLE 2
 β -GALACTOSIDASE ACTIVITY ASSOCIATED WITH RIBOSOMAL PARTICLES OF *E. coli*

Strain	Divisions per minute per O.D. unit at 260 m μ		
	ML 30 (basal)	ML 30 (induced)	ML 308 (constitutive)
	0.017	0.34	0.21
	0.018	0.60	0.17
	0.020	0.46	0.25
	0.016	0.46	0.23
Average	0.018	0.47	0.22

Ribosome fractions were purified by successive centrifugations to constant specific activity and enzyme was assayed with ONPG according to the procedures described under *Methods*.

series of experiments examining the enzymic activity of ribosomes prepared from cultures of noninduced, fully induced, and constitutive cells. The ribosomes were prepared and purified to constant specific enzymic activity by the methods described. It is evident that increased capacity to synthesize enzyme is accompanied by a considerable increase in the amount of ribosome-bound enzyme. A 25-fold increase in the enzymic activity of the ribosome fraction is observed in going from the noninduced to the fully induced state. During this same period, the enzyme content per cell increases by a factor of about 200. It is of interest to note that although the constitutive strain synthesizes about five times as much enzyme per cell as a fully induced cell, this difference is not reflected in an increased amount of ribosome-bound enzyme. On the contrary, this difference was consistently less by a factor of two in the constitutive mutant as compared with the inducible variety.

Discussion.—The experiments described provide strong evidence that the ribosomes carry a small fraction of the β -galactosidase of the cell, possessing three characteristics which distinguish it from the free enzyme molecules: (a) it sediments much more rapidly, possessing a sedimentation coefficient of about 70S; (b) the ribosome-bound activity is increased by exposure to specific antibody; (c) it is not precipitated by an antiserum which removes all of the soluble enzyme present in the reaction mixture. From the results described above, it is not possible to decide whether the enzyme is transiently or permanently associated with the ribosome in the living cell. Experiments to be reported later suggest that both classes are present.

It is of some interest to consider briefly the numerical relations of the partitioning of enzyme molecules between the ribosomal and soluble fractions. For such calculations, we assume that the molecular weight of the RNA in the 70S ribosomes is

1.8×10^6 and that 1 mg of ribosomal RNA/ml will have an optical density of 24. Thus, a solution of O.D. equal to 1 will contain 1.4×10^{13} 70S particles. The turnover number of purified β -galactosidase, measured with orthonitrophenyl- β -D-galactoside, is 4,000 moles/sec per mole of enzyme.⁷ Under the conditions of assay, 1 $m\mu M$ of orthonitrophenol yields an O.D. of 0.000143 at 420 $m\mu$. Using these numbers, one can calculate the number of ribosomes per enzyme molecule from the specific activities of the purified ribosomal preparations. This computation leads to a value of 5×10^3 ribosomes per enzyme molecule for the inducible strain in the noninduced state. Cells growing in C-medium are estimated to contain approximately $5\text{--}10 \times 10^3$ large ribosomes per cell. It is concluded that there is approximately one enzyme molecule per cell bound to the ribosomes in the non-induced strain. This increases by an order of magnitude upon full induction.

The kinetics of this increase are of great interest and are being investigated. The available data suggest that the increase is a fast process completed in about three minutes. The relative rate of enzyme synthesis in constitutive mutants is usually between 5 and 10 times that seen in fully induced inducible strains. It is evident (Table 2) that this higher rate cannot be ascribed to a higher content of ribosomes which can specifically retain β -galactosidase activity.

It must be emphasized that it is difficult to make unambiguous estimations of the absolute activities of the ribosome-bound enzyme molecules. It seems likely, however, that the orders of magnitude computed from these assays can be taken seriously. It is of some interest that the numbers obtained are what might have been expected from a reasonably simple interpretation of ribosome function. If the ribosomes carrying a given protein in association are the only ones concerned with its synthesis, one would conclude that one to ten ribosomes may be active at any given moment in the synthesis of a particular protein. Thus, the several thousand ribosome of an *E. coli* cell could synthesize roughly a thousand different enzymes which should be adequate to carry out the various cell functions. The data described, however, do not eliminate the possibility that a given ribosome may be concerned with the synthesis of a variety of proteins at different times of its existence.

The experiments reported here raise the question of whether other enzymes can be found associated with the ribosomes and whether these also possess similar distinguishing features. Preliminary experiments along these lines in *E. coli* indicate that the same situation obtains with a variety of enzymes. One in particular, alkaline phosphatase, for which an antiserum was available, also exhibited the antibody-activating effect. Halvorson⁸ and his co-workers have uncovered a ribosome-bound fraction of β -glucosidase in yeast. It is again of interest to note that these investigators compute from their data that there is approximately one enzyme molecule bound to the ribosome fraction per cell.

It is evident that the use of specific antiserum may greatly aid the preparation of a ribosome suspension containing a particular protein in bound form and free of its soluble counterpart. Furthermore, the possibility exists of developing a general procedure for isolating ribosomes associated with specific proteins. This is suggested by the experiments in which the ribosome-bound enzyme was precipitated by first complexing with rabbit anti- β -galactosidase and then exposing the complex to an antibody directed against the rabbit γ -globulin.

Summary.—Experiments are described which provide evidence that a certain fraction of the β -galactosidase molecules of the cell are carried on the ribosomes. This fraction corresponds (in order of magnitude) to one molecule per cell in non-induced, inducible cells and rises to between 10 and 20 per cell for the fully induced and constitutive states. In addition to possessing an apparent higher sedimentation coefficient, the ribosome-bound enzyme molecules are distinguishable from their soluble counterparts in their response to specific anti- β -galactosidase serum. Antiserum precipitates the soluble enzyme without affecting the observed activity. Exposure of the ribosome-bound enzyme to antiserum results in a three- to sixfold rise in activity which is not accompanied by the formation of a precipitable aggregate. It was found that the complex can, however, be precipitated by the addition of an antiserum (chick anti-rabbit) directed against the antibody. The latter reaction suggests a means for the isolation of specific ribosomes.

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† This work was done while J. D. Duerksen was a Research Fellow at the Carnegie Institution of Washington. Present address: Department of Microbiology, University of Kansas Medical Center, Kansas City, Kansas.

‡ Aged preparations lose their ability to respond to the specific antibody. One preparation which showed a fourfold increase in the presence of antibody showed no increase after two weeks storage at -20°C .

¹ Hoagland, M. B., in *The Nucleic Acids* (New York: Academic Press, in press), Vol. 3.

² McQuillen, K., R. B. Roberts, and R. J. Britten, these PROCEEDINGS, **45**, 1437 (1959).

³ Bolton, E. T., B. H. Hoyer, and D. B. Ritter in *Microsomal Particles and Protein Synthesis* (New York: Pergamon Press, 1958).

⁴ Britten, R. J., and R. B. Roberts, *Science*, **131**, 32 (1960).

⁵ Elson, D., *Biochim. Biophys. Acta*, **36**, 372 (1959).

⁶ Bolton, E. T., R. J. Britten, D. B. Cowie, B. J. McCarthy, K. McQuillen, and R. B. Roberts, *Carnegie Institution of Washington Year Book*, **58** (1959).

⁷ Cohn, M., *Bacterial Rev.*, **21**, 140 (1957).

⁸ Halvorson, H. O., personal communication (1960).

Comment. See comment on IV.A.3. Richard B. Roberts.

IV.A.3 Ribosomal Enzymes

(Reprinted from Carnegie Institution of Washington Year Book 60, p. 322, 1961.)

J. D. Duerksen and D. B. Cowie

Ribosomal Enzymes

In Year Book 59 the association of a small fraction of the β -galactosidase of a cell with the ribosomes was described. It was found that the quantity of ribosomal enzyme increased considerably upon induction but the kinetics of the increase were not measured.

If this ribosomal enzyme were newly formed, its quantity would provide a measure of the number of active enzyme-forming units in the cell. As the maximal rate of enzyme formation is rapidly established (about $2\frac{1}{2}$ minutes) after the addition of the inducer it was expected that the quantity of ribosomal enzyme might achieve its maximum level with equal rapidity.

Samples were taken at intervals after the addition of the inducer to a culture of growing *E. coli*. They were chilled, washed, and broken, and ribosome pellets were prepared by centrifugation. A second cycle of centrifugation reduced the contaminating soluble enzyme sufficiently so that it did not interfere with the determination of the ribosomal enzyme separated by sedimentation of a layer through a sucrose gradient. The quantity of ribosomal en-

zyme was estimated to be the part of the enzyme that followed the sedimentation pattern of the ribosome. The entire procedure was carried out using 10^{-2} M tris buffer, pH 7.4, with a Mg concentration of 10^{-2} M.

The quantity of ribosomal enzyme (judged by this criterion) continued to rise throughout the induction period. Furthermore, the quantity did not decrease when the inducer was removed. Thus the newly formed enzyme can only be a minor fraction of the total quantity found to sediment at the same rate as ribosomes.

The quantity of ribosomal enzyme was also found to depend markedly on the method of breaking the cells. Cells broken in the French press showed only one-tenth the quantity of ribosomal enzyme that was found when the same culture was broken by grinding with aluminum or by the lysozyme-freeze-thaw technique.

Thus the quantity of ribosomal enzyme depends both on the quantity of enzyme accumulated in the cell and on the method of breaking the cell. Perhaps this indicates that the association is formed at the instant of breakage. In any event, coincidence in sedimentation is not a sufficient criterion to distinguish newly formed enzyme.

Comment. Recent work by Zipser (J. Mol. Biol., in press) has demonstrated one fraction of the ribosomal bound enzyme which is nascent and another which accumulates and is proportional to the enzyme content of the cells. The enzymic activity that he observes as true nascent enzyme associated with ribosomes is equivalent to two molecules of fully active enzyme per cell. Several other examples of nascent enzymes bound to ribosomes have been reported (H. K. Kihara, A. S. L. Hu, and H. O. Halvorson, Proc. Natl. Acad. Sci. U. S., 47, 489-496, 1961; D. Schwartz, Proc. Natl. Acad. Sci. U. S., 48, 750-756, 1962; J. D. Duerksen and M. L. O'Connor, Biochem. Biophys. Res. Commun., 10, 34-39, 1963). Richard B. Roberts.

IV.A.4 Kinetic Studies of β -Galactosidase Induction

Reprinted by permission of The Rockefeller Institute Press, from the Biophysical Journal, November 1961, 1(8), 639-647.

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ABSTRACT The kinetics of β -galactosidase induction in *E. coli* ML 3 have been studied. Following addition of inducer, the rate of enzyme synthesis accelerates from the uninduced to a steady-state rate. At saturating concentration of inducer the time constant (T_e) for this process is 2.5 to 3 minutes. With decreasing inducer concentration (I), increasing time constants are observed. $I/I+K'$ approximates $1/T_e$. The steady-state rate of β -galactosidase synthesis is approximated by P^2/I^2+K^2 . K' and K have been estimated for IPTG and TMG. The kinetics of β -galactosidase production after the removal of inducer by dilution or after the addition of glucose have been investigated. A transition time of 2.5 to 3 minutes is observed before enzyme synthesis slows or stops. These results are consistent with the hypothesis that the enzyme-forming unit is unstable.

INTRODUCTION

The time course of enzyme synthesis following the addition or removal of an inducer provides one of the most important clues to the mechanism of induction. Analysis of induction kinetics has been used frequently to support one or another model of the process (Monod, 1958).

Enzyme synthesis, however, comes after a long and complex sequence of events, any one of which may be rate-limiting. Thus, in some of the early experiments the enzyme was needed to release energy for its own production. Accordingly the time course had predominantly exponential features. This complication can be avoided by the use of inducers which are not substrates, together with an energy supply which is not influenced by the enzyme. The heterogeneous response of the population to induction at less than saturating concentrations of inducer causes another distortion of the induction curve (Novick and Weiner, 1957). This can be eliminated by the use of cells, "cryptic mutants," which lack the concentrating system (Herzenberg, 1959; Cohn and Horibata, 1959b).

When these precautions are followed the rate of enzyme synthesis is rapidly

responsive to changes in the inducer concentration. In fact the response is so rapid that Herzenberg found "no trace of an autocatalytic tendency" (Herzenberg, 1959). This rapidity of the response was cited by Monod as evidence that enzyme induction involved activation of existing templates rather than the synthesis of new ones (Monod, 1958). However, the concept of existing (but not activated) stable enzyme forming units soon encountered serious difficulties in explaining the results of mating experiments (Pardee, Jacob, and Monod, 1959). In these experiments enzyme synthesis achieved its optimal rate within a few minutes after the introduction of DNA from genetically competent cells into genetically incompetent recipients.

The evidence for immediate induction was derived from experiments in which samples were taken at 10 to 20 minute intervals. Accordingly it seemed desirable to repeat these experiments using the better time resolution which can be obtained simply by taking more frequent samples. The results show that a short but readily measurable time interval is, in fact, required before the cells reach their final rate of synthesis. The data support the concept of unstable enzyme-forming units which are rapidly created upon the addition of the inducer.

After these experiments were completed, a paper by Pardee and Prestidge appeared which described a similar series of experiments (Pardee and Prestidge, 1961). Our results are in general agreement with theirs, and provide additional data.

METHODS

Strain. *E. coli* ML 3, a galactoside permease-negative strain of *E. coli* ML 30, was used throughout these experiments.

Medium and Growth Conditions. All experiments were performed with exponentially growing cells vigorously aerated and maintained at 37°C. C medium of the following composition was the only medium employed: 2 gm NH_4Cl , 6 gm Na_2HPO_4 , 3 gm KH_2PO_4 , 3 gm NaCl , 0.01 gm Mg as MgCl_2 , 0.026 gm S as Na_2SO_4 , 10.0 ml 10 per cent maltose, and 900 ml distilled H_2O . Cell density was measured by absorption at 650 m μ using the Beckman DU spectrophotometer. One mg wet weight of cells per ml gives an absorption of 0.400.

Enzyme Assay and Induction of β -Galactosidase. β -galactosidase was determined by the rate of hydrolysis of *o*-nitrophenyl- β -D-galactoside (ONPG). A unit of enzyme is defined as producing 0.1 μM of *o*-nitrophenol per minute at pH 7.2. The thiogalactosides, isopropyl-thio- β -D-galactoside (IPTG) and methyl-thio- β -D galactoside (TMG), both gratuitous inducers, were used to induce the synthesis of β -galactosidase. The inducer was added to exponentially growing cultures at the desired time and concentration. Periodically samples were removed and added to tubes containing 2 drops of toluene. The tubes were then shaken at 37°C for 30 minutes prior to enzymic assay. Under the conditions employed, no correction for the inhibitory effect of the inducers on the enzyme activity was necessary. The IPTG and the TMG used were gifts of Dr. M. Cohn.

RESULTS

General Characteristics of Induction Kinetics. The addition of IPTG or TMG to an exponentially growing culture of *E. coli* ML 3 results in the synthesis of β -galactosidase at an accelerated rate. In Fig. 1 the time course of β -galactosidase induction at a saturating concentration of IPTG is given. The response to the inducer is rapid so that an increase in enzymic activity may be detected within 15 to 30 seconds.

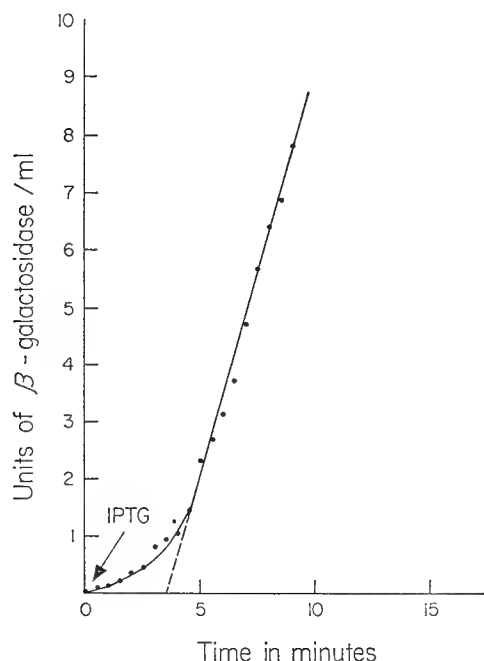


FIGURE 1 Time course of β -galactosidase induction for *E. coli* ML 3.5×10^{-4} M IPTG employed as inducer.

The time constant (T_c) of the transition to the maximum rate of enzyme synthesis from the uninduced level may be obtained by extrapolating the linear portion of the curve to the abscissa (time axis). For the experiment shown in Fig. 1 the time is 3.5 minutes. Slight variations in this time are observed from experiment to experiment with the average being 3.0 minutes. *Times of less than 2.5 minutes have never been observed even when IPTG at a concentration of 10^{-1} M was employed.*

Similar induction kinetics have been observed for ML 30 and 15 TAU⁻, two galactoside permease-positive strains of *E. coli*, using saturating concentrations of inducer.

Effect of Inducer Concentration on Induction Kinetics. Both the steady-state rate of enzyme synthesis after induction and the time required to reach this steady-state rate are dependent on inducer concentration. In Fig. 2 β -galactosidase induction for several concentrations of IPTG is given. As the inducer concentration is decreased from a saturating concentration of inducer the steady-state rate of

enzyme synthesis decreases. The time required to reach this rate, on the other hand, increases with decreasing inducer concentration. In Fig. 2 the quantity of enzyme is plotted against bacterial mass. This procedure is convenient to determine accurately the steady-state rates of enzyme synthesis especially in those cases in which measurements must be made over several generations.

The time constant, T_c , may be obtained from such a plot by extrapolating the linear portion of the curve to the basal enzyme level and converting the corre-

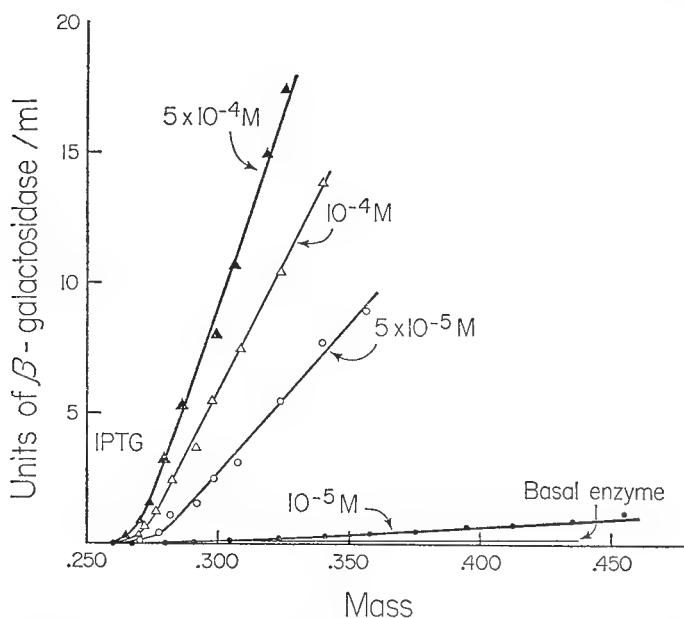


FIGURE 2 Induction of β -galactosidase at several concentrations of IPTG. Basal enzyme is the uninduced level. Bacterial mass is given as the absorption at $650\text{ m}\mu$. 1 mg wet weight of cells per ml corresponds to an absorption of 0.400.

sponding change in bacterial mass to time. When the steady-state rate of β -galactosidase synthesis is fast compared to the basal rate, extrapolation to either the abscissa or the basal enzyme level gives approximately the same constant for induction. However, when the rate is slow, extrapolation must be made to the basal enzyme level rather than to the abscissa to avoid considerable error in determining the time constant.

In Fig. 3 the effects of various IPTG concentrations on the time constant and on the steady-state rate of β -galactosidase synthesis are summarized on a log-log plot. This is a convenient procedure for determining the relationship between these two parameters and the inducer concentration. In each case the experimental curves were compared with theoretical curves describing various functions of I , the inducer concentration.

A function of I that fits the experimental data on the effect of IPTG concentration

on the reciprocal of the time constant is $I/I + K'$. From the slope of the curve K' may be shown to be 5×10^{-5} moles per liter. A fit for the data showing the effect of IPTG concentration on the steady-state rate of enzyme synthesis is given by the function $I^2/I^2 + K^2$ where K is 1×10^{-4} moles per liter.

In Table I, the effects of TMG concentration on the rate of enzyme synthesis and on the time constant are summarized. A log-log plot of these data shows that at high TMG concentration the rate of β -galactosidase is approximated by $I^2/I^2 +$

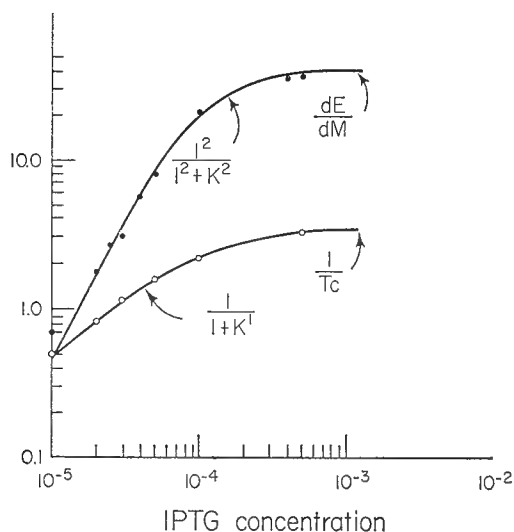


FIGURE 3 Log-log plot of the effect of various IPTG concentrations on the steady-state rate of β -galactosidase synthesis and on the time constant. Experimental data presented as circles. Solid circles describe effect on rate of synthesis. The corresponding line is theoretical curve of $I^2/I^2 + K^2$. Open circles describe the effect of inducer concentration on $1/T_c$ with the corresponding line that of $I/I + K'$. IPTG concentration is moles per liter.

K^2 where K equals 3.5×10^{-3} moles per liter but deviates from the theoretical curve at low inducer concentration. The reciprocal of the time constant fits the equation $I/I + K'$ where K' is 3×10^{-4} moles per liter.

Kinetics of Enzyme Production after Removal of Inducer. The synthesis

TABLE I
THE EFFECT OF TMG CONCENTRATION ON THE
STEADY-STATE RATE OF β -GALACTOSIDASE SYNTHESIS
AND ON THE TIME CONSTANT OF INDUCTION

TMG	dE/dM^*	T_c min.
1×10^{-2} M	39.9	3
2×10^{-3} M	11.7	3
1×10^{-3} M	5.1	4
1×10^{-4} M	0.34	12
5×10^{-5} M	0.16	22

* dE/dM = steady-state rate of β -galactosidase synthesis, given as the change in the number of enzyme units per increase of $250 \mu\text{g}$ wet weight of cells.

of β -galactosidase at induced rates stops after the removal of the inducer. However, the cessation of enzyme production is not immediate, as previously reported (Cohn, 1957); there is a short transition period after the removal of inducer before enzyme synthesis at induced rates ceases. The transition is conveniently studied using a culture of *E. coli* ML 3 in which the inducer concentration is suddenly decreased to a low level by dilution. In Fig. 4 the time course of enzyme production following dilution of the IPTG concentration from 3×10^{-5} M to 1×10^{-5} M is given. This dilution causes the rate of enzyme production to decrease by a factor of 4. A transition period of approximately 3 minutes is observed before the new rate of synthesis is established. There is no preinduction effect (Monod, 1956). The rate

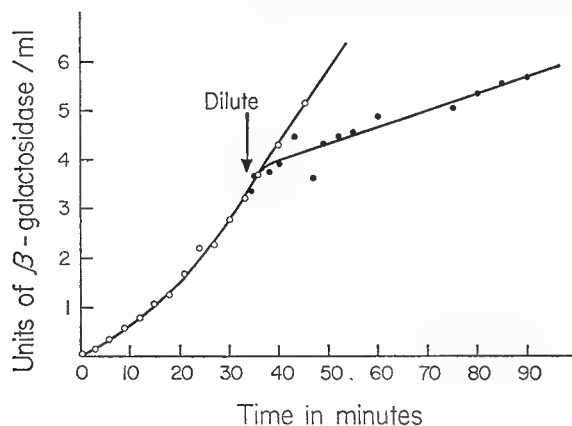


FIGURE 4 The kinetics of β -galactosidase production after dilution of the inducer concentration. Induction was initiated by the addition of 3×10^{-5} M IPTG at $T = 0$. After 34 minutes, an aliquot of the culture was diluted threefold. Solid circles are measures of the enzyme production in the diluted culture. Values have been multiplied by 3 for presentation on this graph.

established in the presence of 1×10^{-5} M IPTG after dilution is the same as that observed when the induction is initiated by the addition of that concentration of inducer to an uninduced culture of cells.

The time during which the rate of enzyme synthesis deaccelerates from one rate to another is independent of the time required to reach the faster rate from a lower one, thus suggesting different mechanisms for the two processes. For example, a transition time of approximately 3 minutes is observed when the concentration is diluted from 3×10^{-5} M to 1×10^{-5} M (Fig. 4). However, a period of about 8 minutes is required to achieve the faster rate when the IPTG concentration is raised from 1×10^{-5} M to 3×10^{-5} M.

The Glucose Effect. The stock of *E. coli* ML 3 used in these experiments is "glucose-insensitive," i.e., galactosidase may be induced to the maximal level in a culture growing exponentially on glucose. The kinetics of induction, however, are altered by the presence of glucose.

If 10^{-2} M glucose is added simultaneously with 5×10^{-4} M IPTG, an effect on the time constant of induction but not on the final steady state rate of enzyme production, is observed (Fig. 5). The time constant for induction is lengthened from 3 minutes to approximately 13 minutes by the addition of glucose. By decreasing the inducer concentration from 5×10^{-4} M to 5×10^{-5} M, an effect of glucose not only on the time constant but also on the steady-state rate may be observed.

If glucose is added after the induced synthesis of β -galactosidase has been initiated, enzyme production stops or slows for a short time and then returns to the maximal rate. In one of the experiments shown in Fig. 5, 10^{-2} M glucose was

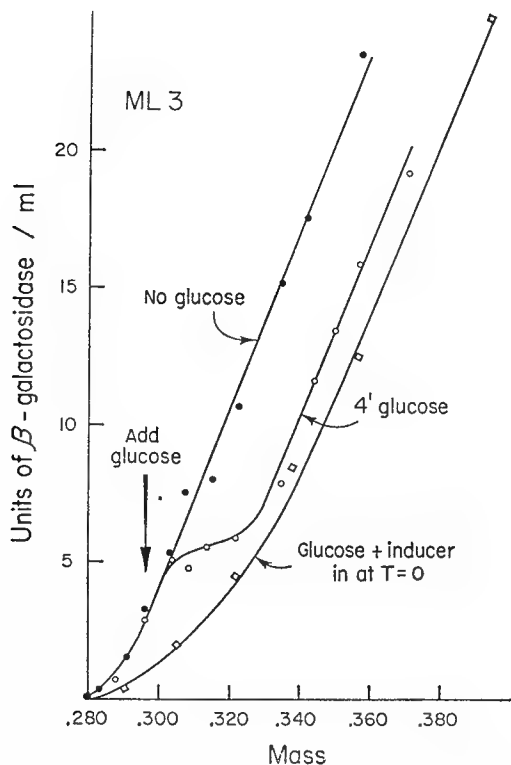


FIGURE 5 The effect of glucose on β -galactosidase induction. The induction was initiated by the addition of 5×10^{-4} M IPTG at $T = 0$ (mass = 0.280). 10^{-2} M glucose was added simultaneously with inducer (\square) or 4 minutes after the inducer (\circ). No glucose was added in the control (\bullet). Bacterial mass is given as absorption at 650 $m\mu$. 1 mg wet weight per ml corresponds to an absorption of 0.400.

added 4 minutes after the addition of 5×10^{-4} M IPTG. The rate of enzyme production fell, after the addition of the glucose, to one-eighth the maximal rate with a transition time of about 2.5 minutes. Six minutes later the maximal rate was reestablished.

The slowing down of β -galactosidase synthesis after the addition of glucose is not due to a general effect on the culture. Growth as measured by absorption continues at the same rate and the incorporation of radiosulfur into proteins is unaffected. Also the reestablishment of the maximal rate of synthesis is not due to depletion of the glucose. It is present in a concentration high enough to provide growth for several generations. The time required for the enzyme production to

slow to the new rate is the same as that observed after the removal of inducer by dilution (Fig. 4).

DISCUSSION

The time course of induction at any particular concentration of inducer measures two quantities of theoretical interest; the final steady-state rate of enzyme synthesis (S) and the time constant of the transition to the new rate (T_c). In the mutant ML 3 the rate does not depend on the past history of the cells.

When the rates are plotted as a function of the inducer concentration a saturation curve results (Fig. 3). For the inducer IPTG,

$$S \sim I^2/I^2 + K^2$$

This relationship indicates that reactions requiring two molecules of inducer determine the rate of enzyme synthesis.

The cause of the delay in enzyme synthesis is not immediately obvious. Képès has studied the time required for the accumulation of inducers in various strains of *E. coli* (Képès, 1960). With ML 3 blocked by chloramphenicol the time constant for the accumulation of TMG at 26° was ~ 6 minutes. In his experiments it was necessary to wash the cells so that his results pertain only to that fraction of the TMG which was not readily removed by washing. Pardee and Prestidge, on the other hand, have carried out numerous experiments which indicate that the time required for the entry of the inducer does not limit the rate of enzyme induction (Pardee and Prestidge, 1961).

In the experiments reported here it is found that the time constant T_c varies with the concentration of the inducer as shown in Fig. 3:

$$\frac{1}{T_c} \sim \frac{I}{I + K'}$$

Such a relationship would not be expected if the delay in reaching the final rate of enzyme synthesis were due to the time required for the inducer to penetrate the cell. If this were the case no delay should be observed at high or saturating concentrations of inducer. Furthermore, higher concentrations of inducer should be required to give minimal delays than are required to give maximal rates of synthesis. In our experiments concentrations of inducer which give minimal delays are not sufficient to produce maximal rates of synthesis. It is concluded, in agreement with Pardee and Prestidge, that the entry of the inducer is not a rate-limiting factor.

When the steady-state rate of enzyme synthesis is reduced by dilution of the inducer or by the addition of glucose, the time constant of the transition to the lower rate is 2.5 to 3 minutes. This time constant is the same as the time constant for induction at saturating concentrations of inducer. For these transitions the time constant should be determined by the time constant for the decay of the enzyme-

forming unit. This is in agreement with Pardee and Prestidge that the enzyme-forming units are unstable. These results indicate that the enzyme-forming units decay with a time constant of 2.5 to 3 minutes.

The rate of enzyme synthesis and the time constant for induction show quite different dependences on the kind of inducer employed (whether TMG or IPTG) and on the concentration of these inducers. It therefore seems necessary to envisage that induction involves two different reactions involving the inducer molecules. One of these reactions influences the rate of synthesis and the other affects only the time constant.

A further indication of the existence of two separate reactions is found in the temporary inhibition caused by glucose. The repression is of brief duration and quite unlike the permanent repression usually reported (Cohn and Horibata, 1959a) Képès observed that glucose caused a reduction in the level of intracellular TMG (Képès, 1960). However, he found no indication that the original level was reestablished. Thus his findings might pertain more to the situation in which the repression was permanent. Alternatively the addition of glucose might cause the temporary accumulation of a repressive derivative, but such a product would not be expected to be completely removed after a short time interval even though an ample supply of glucose remained. It seems more likely that the glucose affected the reaction controlling the time constant without effect on the reactions controlling the final rate.

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IV.A.5 A Model for the Mechanism of Enzyme Induction

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ABSTRACT A sequence of reactions is postulated from which are derived equations describing the time course of enzyme induction. The model also yields the observed effect of the inducer concentration on the time constant and final rate of enzyme synthesis. Features of the model are: (a) The inducer acts first to release the protein forming template from its site of synthesis on the gene. (b) The inducer is involved again in the equilibrium dissociation of the free template-inducer complex which is utilized in the synthesis of the enzyme-forming unit. (c) The final enzyme-forming unit is unstable and must be synthesized continuously to maintain enzyme synthesis.

INTRODUCTION

Many attempts have been made to deduce the underlying mechanism of enzyme induction from measurements of the time course of enzyme synthesis. The data available have not been adequate for this purpose, however, because in some cases the process of interest was obscured by other complicating factors, and in other cases the time resolution was not sufficient to follow the rapid changes of synthetic rates.

The experiments of Boezi and Cowie (1961) provide data in which these difficulties appear to be lacking. Thus they furnish the material for one more attempt to uncover the type of reactions which are involved. Furthermore the data cover a number of experimental situations which provide a rigorous test of any postulated reaction mechanism.

The experimental data are the quantities of cells and of enzyme, measured as a function of time, through a period when the concentrations of inducers or repressors vary and the cells respond by changes in their rate of enzyme synthesis. Since the enzyme itself is stable, at least for the duration of the experiments, the quantity of theoretical interest is $(dE/dt)/Q$, the average rate of enzyme synthesis per cell. This quantity can be considered to be a measure of the number of active enzyme-forming units per cell (N). Thus all enzyme-forming units are assumed to be fully

active; the possibility that enzyme-forming units may have different rates of synthesis is ignored. Relative values of N can be determined readily from the experimental data; conversely, if N can be predicted by theory, the expected quantity of enzyme can be calculated for comparison with experimental data. This procedure is valid only when the experiments show that $(dE/dt)/Q$ does not depend upon E/Q , the quantity of enzyme accumulated.

REACTION SEQUENCE

The experimental data show that upon induction N approaches the steady-state level, N_s , as shown in equation (1).

$$N = N_s(1 - ge^{-\alpha t} - he^{-\beta t}) \quad (1)$$

where $g + h = 1$.

Furthermore, the data show that

$$N_s \sim \frac{I^2}{I^2 + K_2^2}$$

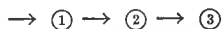
and

$$\beta \sim \frac{I}{I + K_4}$$

where I is the concentration of inducer.

The two separate exponential terms are required to allow a long time constant for induction at low levels of inducer and a rapid loss of N when the inducer is diluted out.

Such an equation also describes the appearance of material in the second component of a three component sequence of reactions:



Thus the form of the induction curve suggests that: (a) The enzyme-forming unit is not a stable end product but is converted into an inactive form. (b) The enzyme-forming unit is derived from a pool of precursor material which must be accumulated.

One sequence of reactions which leads to the proper form of the induction curve is the following:



Reaction (2) indicates that a precursor material, X , accumulates on a template, A , and is polymerized to form a product, B , which remains complexed with the template, A .

Reaction (3) is a reversible reaction of the complex AB with two molecules of inducer, I .

Reaction (4) represents the dissociation of the complex, ABI_2 . This releases the product, BI_2 , and frees the template, A , for further synthesis.

Together Reactions (2), (3), and (4) determine the inducer dependence of the steady-state level of N but have no influence on the time constant.

Reaction (5) is a reversible dissociation of BI_2 and Reaction (6) is the association of the complex, BI_2 , with some cellular component, Y , to make the enzyme-forming unit N . Reaction (5) introduces the inducer dependent term into the time constant without introducing any further inducer dependence in N_s .

Reaction (7) indicates that the enzyme-forming unit is unstable and disintegrates into inactive products, Z .

This system of reactions may be studied most conveniently by noting that the first three provide the mechanism of producing BI_2 , while the next two are concerned with its fate.

First, let us find a relationship for the production of BI_2 by the first three reactions. We shall assume that X and I are present in sufficient quantity so that their concentrations are not significantly altered by the reactions. We shall also assume that the usual "steady-state" approximation will be adequate. This simply means that the reaction system adjusts to a change of conditions sufficiently rapidly so that the intermediates are always at their steady-state concentrations, or, in other words, that transients are quickly damped out. Then we have for the rate of production of BI_2 ,

$$R_{BI_2} = k_3(ABI_2)$$

with the conditions:

$$A_T = (A) + (AB) + (ABI_2) \quad \text{conservation of } A$$

$$k_1(X)(A) = k_3(ABI_2) \quad \text{steady state for } A$$

$$k_2''(AB)(I)^2 = k_1(X)(A) + k_2'(ABI_2) \quad \text{steady state for } AB$$

where A_T is the total available A in the system. Thus

$$R_{BI_2} = K_3 A_T \frac{(I)^2}{(I)^2 + K_2^2},$$

where we define

$$K_3 = \frac{k_1(X)k_3}{k_1(X) + k_3}$$

and

$$K_2^2 = \frac{k_1(X)[k_3 + k_2']}{k_2''[k_1(X) + k_3]}.$$

Now, turning to the question of the time dependence of BI_2 we note that

$$\frac{d[(BI_2) + (BI)]}{dt} = R_{BI_2} - k_5(Y)(BI_2).$$

We now assume that Y is available in sufficient excess so that its concentration is not sensibly time-dependent and that Reaction (5) is sufficiently fast so that it is always essentially at equilibrium, *i.e.*,

$$(BI) \simeq \frac{K_4}{(I)} (BI_2) \quad \text{with} \quad K_4 = k_4''/k_4'.$$

Then we have

$$\frac{K_4 + (I)}{(I)} \frac{d(BI_2)}{dt} = R_{BI_2} - k_5(Y)(BI_2)$$

which integrates, with the initial condition that $(BI_2) = 0$ at $t = 0$, to give (BI_2) as a function of time. This is now substituted in the equation for the number of enzyme-forming sites, N ,

$$\frac{dN}{dt} = k_5(Y)(BI_2) - k_6N,$$

which is integrated, with the initial condition $N = 0$ at $t = 0$, to give

$$N = N_s \left[1 - \frac{\beta}{\beta - \alpha} e^{-\alpha t} - \frac{\alpha}{\alpha - \beta} e^{-\beta t} \right]$$

where

$$N_s = \frac{K_3 A_T}{k_6} \frac{(I)^2}{(I)^2 + K_2^2}$$

$$\alpha = k_6$$

$$\beta = k_5(Y) \frac{(I)}{(I) + K_4}$$

Thus we have obtained an equation for N of the form called for in equation (1).

To compare with the actual data we must now find the concentration of the enzyme, E . Let us consider the case where the times involved are sufficiently short that the cell density, Q , is essentially unchanged. Then

$$\frac{dE}{dt} \sim QN$$

which integrates, with the initial condition $E = 0$ at $t = 0$, to give

$$E \sim QN_s \left[t + \frac{\beta(e^{-\alpha t} - 1)}{\alpha(\beta - \alpha)} + \frac{\alpha(e^{-\beta t} - 1)}{\beta(\alpha - \beta)} \right].$$

This equation predicts that, after a "time lag" there will be a linear rise in enzyme concentration and the rate will be proportional to $(I)^2/[(I)^2 + K_2^2]$, in agreement

with Boezi and Cowie. If the linear portion of the curve is extrapolated back to $E = 0$, then it will intersect the abscissa at a time T_c given by

$$T_c = \frac{\alpha + \beta}{\alpha\beta}.$$

Thus

$$\begin{aligned} \frac{1}{T_c} &= \frac{k_6 k_5(Y)(I)}{k_6 K_4 + [k_6 + k_5(Y)](I)} \\ &\sim \frac{I}{I + K'}, \end{aligned}$$

again in agreement with Boezi and Cowie.

In the more general case there will be some cell growth during the process and this must be taken into account in integrating to find the enzyme concentration. The method is given in the Appendix.

The reaction sequence postulated was based solely upon the kinetics of induction and the loss of induction upon dilution of the inducer. It was gratifying therefore to find that the same reaction sequence provided a mechanism for the temporary repression caused by glucose. When the inducer concentration is high, glucose affects the time constant of induction but not the final rate of synthesis. It appears to compete with the inducer in Reaction (5) but not in Reaction (3). Thus the complex AB seems to show a greater specificity than does BI .

When glucose is added to cells which are already induced there is a temporary depletion of BI_2 by glucose substitution for one I , thereby shutting off the production of enzyme-forming units. Those already present decay and the rate of enzyme synthesis drops exponentially. Glucose does not influence the production of new BI_2 ; hence BI accumulates until the level of BI_2 is restored.

At higher ratios of glucose to inducer the rate of enzyme synthesis is also reduced, presumably by competition in Reaction (3).

The kinetics of induction using TMG in place of IPTG are correlated by making suitable changes in K_2 and K_4 . K_2 must be decreased by a factor of 35 and K_4 by a factor of 6. Again Reaction (3) shows a greater specificity than does Reaction (5).

LIMITATIONS OF THE MODEL

These equations are by no means unique. In particular there is no way to distinguish by kinetic analysis whether the inducer plays a direct active role as indicated or whether the inducer inactivates or removes a repressor. The form of the equations can remain unchanged when written in terms of interactions between inducer molecules and a hypothetical repressor.

Furthermore, these reactions are undoubtedly too simple and represent only those reactions which predominate in the range of experimental conditions tested. For example, Reaction (5) indicates the partial dissociation of a complex contain-

ing two molecules of inducer but the complete dissociation (*i.e.*, $BI \rightleftharpoons B + I$) is neglected. Similarly the possibility that one molecule of *I* might strip *B* off *A* is ignored. The divergence between the theoretical and experimental curves at very low concentrations of inducer may well be due to such omissions. The effective concentration of the inducer is assumed to be the external concentration. The time required for the inducer to penetrate the cell is ignored.

Neither have we found it necessary to include any additional reaction of the inducer with the enzyme-forming unit itself. Such a reaction might well be present but would have no influence on the experimental data if it saturated at extremely low concentrations of inducer. In short, we believe that these reactions represent one of the simplest formulations adequate to describe the prominent features of induction of β -galactosidase in the mutant ML 3. In other systems, other reactions might become rate-limiting.

IDENTIFICATION OF THE CELLULAR COMPONENTS

The reactions postulated become much more meaningful if the symbols *A*, *B*, *etc.* can be associated with known cellular components. However, this correlation is quite independent of the validity of the equations themselves and is much more speculative. The correlations may be quite wrong even though the equations are correct.

It seems quite certain that *A* represents DNA. Pardee *et al.* (1959) have shown that induction is initiated when genetically competent DNA enters a cell. It seems equally certain that *N* must represent the 70S ribosomes as they have been shown to be the principal sites of protein synthesis and to carry a small portion of the enzyme (McQuillen *et al.*, 1959; Cowie *et al.*, 1961).

B and *Y* are then the components needed to form an active protein-synthesizing unit. *B*, the unit formed on the DNA template, has the characteristics of the "messenger RNA" postulated by Jacob and Monod (1961). Accordingly *Y* must represent the bulk of the active 70S particle. Presumably an ample supply of *Y* exists before induction because the time course of ribosome synthesis is slow compared to the rapidity of enzyme induction. *Y* must therefore be non-specific or only partially specific.

Alternatively *B* could represent an early stage of ribosome synthesis during which these precursors of the ribosomes acted as templates for protein synthesis. The half-life of the first precursor corresponds closely to the half-life of the enzyme-forming unit (Britten, 1961).

Z represents the inactive particles after the "messengers" are destroyed or the ribosome precursors are converted to ribosomes.

Since *B* combines specifically with the inducer it is attractive to postulate that *B* also carries at least the active site of the enzyme. If so, *X* must represent the amino acid precursors of the enzyme in addition to the nucleotide precursors of the RNA.

CONCLUSION

We have no confidence that this model will survive long. It is quite definite and therefore highly vulnerable to experimental disproof. It does, however, unify a wide variety of experimental data. Furthermore it indicates an additional reaction which might take part in the regulation of enzyme synthesis, namely in the association of the newly formed RNA with an existing particle to create an enzyme-forming unit. This association could be blocked or facilitated by alterations in the protein of the ribosomes and thus controlled by genes remote from the genes determining the structure of the enzyme.

APPENDIX

In the case of experiments covering a time long enough to necessitate correcting for cell growth, a somewhat different analysis is required. In this case

$$Q = Q_0 e^{at}$$

and

$$\frac{dE}{dt} \sim QN,$$

using N as before, now integrates, with $E = 0$ at $t = 0$, to give

$$E \sim N_0 Q_0 \left[\frac{e^{at} - 1}{a} + \frac{\beta \{e^{-(\alpha-a)t} - 1\}}{(\beta - \alpha)(\alpha - a)} + \frac{\alpha \{e^{-(\beta-a)t} - 1\}}{(\alpha - \beta)(\beta - a)} \right].$$

This may better be represented, for comparison with experimental data, by replacing e^{at} by Q/Q_0 to get

$$E \sim N_0 \left[\frac{Q - Q_0}{a} + \frac{\beta \{e^{-\alpha t} Q - Q_0\}}{(\beta - \alpha)(\alpha - a)} + \frac{\alpha \{e^{-\beta t} Q - Q_0\}}{(\alpha - \beta)(\beta - a)} \right]$$

If E is now plotted against Q , there will appear to be a "lag" and then E will grow linearly with Q according to the relation

$$E \sim N_0 \left[\frac{Q - Q_0}{a} - \frac{BQ_0}{(\beta - \alpha)(\alpha - a)} - \frac{\alpha Q_0}{(\alpha - \beta)(\beta - a)} \right].$$

Extrapolating the linear part back to $E = 0$, we will find an intercept on the Q axis at Q_e . Then

$$\frac{Q_e - Q_0}{aQ_0} = \frac{\alpha + \beta - a}{\alpha\beta - (\alpha + \beta)a + a^2}.$$

Incidentally, it may be noted that in the limit as $a \rightarrow 0$, all these equations reduce to the results given earlier for the case of no growth.

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IV.B.1 The Effects of 6-Mercaptopurine on Biosynthesis in Escherichia coli

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The purine analogue, 6-mercaptopurine (6-MP) (1), has been the subject of considerable study, but the mechanisms of its actions are still not clear. This drug inhibits the proliferation of acute and chronic myelocytic leucemias (2) and the growth of bacteria (3). It interferes with the utilization of formate for nucleic acid synthesis (4) and suppresses the formation of adaptive enzymes (5). However, it has not been shown whether the various biochemical effects of the drug could be observed in a single species, or whether the biochemical effects reflected one or many sites for the drug's action. Therefore, a systematic study of the effects of 6-MP on the biosynthesis in *Escherichia coli* B was undertaken. This strain of bacteria has been the subject of extensive biochemical investigations (6).

A preliminary report on the results of these experiments has been presented (7).

Materials

6-MP was obtained from Burroughs Wellcome and Company, Tuckahoe, New York, courtesy of Dr. G. H. Hitchings. Aliquots of solutions of 6-MP (1 mg. per ml.) in 0.1 per cent Na_2CO_3 were added to bacterial cultures as required. Unless otherwise specified, the concentration of the drug in the medium was 10 γ per ml. Radioactive sulfate and phosphate were obtained from the Atomic Energy Commission at Oak Ridge, Tennessee. C^{14} -Formate and C^{14} -formaldehyde were purchased from the Isotopes Specialties Company, Inc., Glendale, California, and 1- C^{14} -acetate from Tracerlab, Inc., Boston, Massachusetts. 8- C^{14} -Adenine and 2- C^{14} -uracil were products synthesized as described previously (8, 9). Glucose-salts media as described by Roberts *et al.* (6) were used for culturing the bacteria. *E. coli* B (ATCC 11303) was obtained from the American Type Culture Collection, Washington, D. C.

Methods

Growth of Bacteria—*E. coli* cells were grown in 500 ml. polyethylene bottles and aerated by mechanical shaking at 37°. Exponentially growing

cultures were sampled through a spout into cuvettes of a Beckman model DU spectrophotometer merely by squeezing the flexible bottles. Bacterial growth was represented by the increase in optical density at 650 m μ ($\Delta\rho$), *i.e.* the difference between the measured optical density (ρ) and that at the beginning of the experiment (ρ_0). Viable bacterial counts of normal and 6-mercaptopurine-inhibited cultures compared at selected optical densities showed no differences between the two cultures, justifying the use of optical density measurements as criteria of growth (10). When required, aliquots were withdrawn from the Beckman cells and further analyzed.

Chemical Fractionation—Bacteria were harvested and washed in the centrifuge and fractionated by a modification (6) of the Schneider (11) procedure.

When chemical estimations of the bacterial nucleic acid content were required, the cells were extracted successively with cold 5 per cent trichloroacetic acid (TCA), 80 per cent ethanol, and boiling 10 per cent NaCl (12) for 30 minutes to release nucleic acids. The absorption of light at 260 m μ by the 10 per cent NaCl extract was taken as a measure of the nucleic acid content.

Kinetic studies of the utilization of labeled compounds were made by using the technique developed by Britten, Roberts, and French (13). Bacterial samples were withdrawn at intervals, and the optical density was measured. 2 ml. of each sample were added to an equal volume of TCA at room temperature, and the resulting suspension was filtered through Schleicher and Schuell membrane filters and washed with 5 ml. of 5 per cent TCA. After drying, the filters were assayed for radioactivity.

Radioactivity Measurements—All radioactivity determinations were made with a thin mica, end window counter, by using the procedures previously described (6).

Results

Effect of 6-MP on Bacterial Growth—Fig. 1 shows the growth curves of a control culture of *E. coli* (Curve A) and a parallel culture treated with 10 γ of 6-MP per ml. (Curve E). 6-MP decreased the growth rate of the bacteria within a few minutes after its addition to the cultures.

Since vitamins and coenzymes are known to promote the growth of bacteria or to interfere competitively with growth inhibition brought on by drug treatment (14), a number of substances and mixtures were tested for their ability to reverse the inhibitory effect of 6-MP. Coenzyme A, folic acid, biotin, niacin, riboflavin, a mixture of the B vitamins, adenosine triphosphate, thiamine, pyridoxine, vitamin B₁₂, glutathione, casein hydrolysate, a mixture of diphosphopyridine nucleotide, asparagine, and gluta-

mine; carbamyl phosphate, and uracil were without effect upon the growth curves of 6-MP-inhibited bacteria.

Hypoxanthine, adenine, or guanine added to cultures of 6-MP-inhibited bacteria restored the growth rate to that of the control cultures. The effect of adenine on the growth rates of 6-MP-inhibited cultures is also shown in Fig. 1. The simultaneous addition of adenine and 6-MP to a bacterial culture prevented the inhibitory effect of the latter compound. If the cells were allowed to grow for 10, 30, or 60 minutes in the presence of the drug and were then treated with adenine, reversal of inhibition took

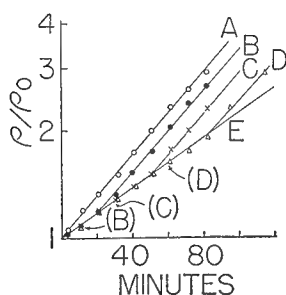


FIG. 1

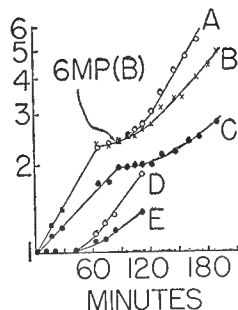


FIG. 2

FIG. 1. Inhibition of bacterial growth by 6-MP and reversal of growth inhibition with adenine. Curve A, no 6-MP; Curves B, C, and D, 6-MP added at 0 minute, adenine added at 10, 30, and 60 minutes, respectively; Curve E, 6-MP added at 0 minute, no adenine. $\rho:\rho_0$ = ratio of optical density at any time to the optical density at 0 minute.

FIG. 2. 6-MP inhibition of bacterial growth during adaptation to lactose. 10 mg. of glucose and 20 mg. of lactose added to cultures of Curves A, B, and C. Curve A, no 6-MP; Curve B, 6-MP added at 88 minutes; Curve C, 6-MP added at 0 minute. 20 mg. of lactose added to cultures of Curves D and E. Curve D, no 6-MP; Curve E, 6-MP added at 0 minute.

place only after a considerable delay (Fig. 1, Curves B, C, and D), which was directly related to the period of exposure of the cells to 6-MP. 6-MP, therefore, specifically affected the biosynthesis of cell components from purines.

Reversal could be achieved also by subculturing 6-MP-treated cells in 6-MP-free culture media. Furthermore, when the medium concentration of 6-MP was 1.5 γ per ml. or less, the growth rate returned to that of the control after 2.5 hours of inhibition. It is evident, therefore, that the cell damage caused by 6-MP was not permanent.

Effect of 6-MP on β -Galactosidase Synthesis—The production of the inducible enzyme, β -galactosidase, was examined to determine whether 6-MP influenced the extent of biosynthesis of a specific protein. *E. coli*

cells growing exponentially with glucose as the energy source were harvested, washed, and resuspended in a culture medium lacking an energy source. 50 ml. aliquots of the suspension were placed in each of five culture bottles. A mixture of 10 mg. of glucose and 20 mg. of lactose was added to each of three of the suspensions (Fig. 2, Curves A, B, and C). 20 mg. of lactose were added to the remaining two cell suspensions (Fig. 2, Curves D and E). No 6-MP was added to the control cultures (Curves A and D). 0.5 mg. of 6-MP was added to the cultures represented by Curves C and E at the same time that the energy sources were added and to the culture of Curve B when the growth curve indicated that the glucose of the culture was exhausted. Optical densities of each culture were measured periodically, and aliquots, after injection into a solution of chloramphenicol (50 γ per ml.) to prevent further enzyme synthesis, were analyzed for β -galactosidase essentially by the method of Koppel *et al.* (15).

Curve A of Fig. 2 followed the typical diauxic growth pattern expected for cultures growing on the glucose-lactose combination (16). Curve B followed Curve A (the control), until the addition of 6-MP. Thereupon the rate of growth decreased, reaching finally the typical 6-MP-inhibited rate. The cells of the culture of Curve C grew at the 6-MP-inhibited rate during the metabolism of glucose and, after adaptation, lactose. Curves D and E show that 6-MP inhibited the growth of bacteria when lactose alone provided the energy source. The β -galactosidase determinations showed that the enzyme was formed after the bacteria had exhausted the glucose and were growing on lactose whether or not the cultures contained 6-MP. The enzyme content was found to be proportional to the amount of growth on lactose irrespective of the presence of 6-MP. It is concluded that 6-MP inhibited the synthesis of the adaptive enzyme β -galactosidase only in accord with its inhibitory effect upon cell growth.

Utilization of Radioactive Sulfate—The uptake of radioactive sulfate was used as a measure of total protein synthesis. A control culture of bacteria and a culture inhibited with 6-MP were grown in the glucose-salts media to which had also been added $S^{35}O_4^{=}$. Samples were withdrawn at intervals from each culture, and the TCA-extracted cell residues containing the protein sulfur of the bacteria (17) were analyzed for radioactivity. The data are presented in Fig. 3, where the uptakes of S^{35} by a control and a treated culture are compared for similar extents of growth. By this means of presentation the general decrease in tracer uptake expected as a result of the slower growth of inhibited cells is compensated, and specific effects become apparent. The results for the control and the 6-MP-inhibited culture were clearly the same. Since the optical density of the culture is proportional to the bacterial mass (6) and since the incorporation of sulfate sulfur is directly proportional to the amount of new protein

synthesized (17), it is concluded that 6-MP influenced the utilization of sulfate sulfur for protein synthesis only in accord with its effect upon the rate of bacterial growth. These results are in agreement with those for β -galactosidase.

Utilization of 8-C¹⁴-Adenine, 2-C¹⁴-Uracil, and C¹⁴-Formate—These compounds contribute carbon chiefly to the nucleic acids of growing *E. coli* (6). Since 6-MP was suspected to interfere with nucleic acid synthesis (3, 18), it was of interest to examine the incorporation of these nucleic acid precursors in the presence of the drug by using the membrane filter technique. Since adenine reversed the effects of 6-MP, no effect upon the uptake of C¹⁴-adenine was expected, and none was observed. Fig. 4

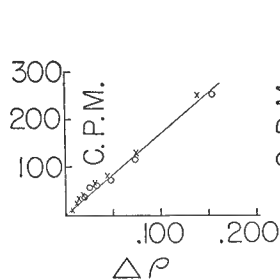


FIG. 3

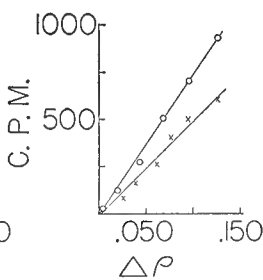


FIG. 4

FIG. 3. Utilization of $S^{35}O_4^{2-}$ by control (O) and 6-MP-inhibited (X) *E. coli*. Radioactivity is expressed in counts per minute (c.p.m.) and growth as the increase in optical density ($\Delta\rho$).

FIG. 4. The utilization of C¹⁴-formate by control (O) and 6-MP-inhibited (X) *E. coli*.

shows the inhibitory effect of 6-MP on the utilization of C¹⁴-formate by *E. coli*. The uptake of formate carbon is directly proportional to the amount of new growth ($\Delta\rho$) whether or not 6-MP is present. In the presence of the drug, however, the uptake of C¹⁴ is *less per unit growth* than in the control culture. These results agree with those of Skipper (4) regarding the inhibition by 6-MP of the *de novo* synthesis of purines from labeled formate in the mouse. 6-MP also suppressed the utilization of exogenous 2-C¹⁴-uracil to a greater extent than expected as a result of mere growth inhibition.

To determine the effect of non-radioactive adenine supplementation on 2-C¹⁴-uracil utilization by bacteria treated with 6-MP, two bacterial cultures were prepared as above with 2-C¹⁴-uracil. 6-MP was added to one of the cultures, and the membrane filter analysis was carried out. Non-radioactive adenine (10 γ per ml.) was then added to both cultures after 70 minutes, and the analysis was continued. The results (Fig. 5) show

initially the characteristic depression in the uptake of uracil in the case of the 6-MP-treated bacteria. Following the addition of adenine, however, there was an increase in the amount of C^{14} -uracil utilized per unit of new growth until the slopes of the uptake curves became the same for both cultures. It is apparent that adenine reversed the 6-MP-induced inhibition of uracil uptake as well as of growth.

The results with the labeled nucleic acid precursors suggest that one of the effects of 6-MP upon bacterial metabolism is the suppression of nucleic acid synthesis. This hypothesis was directly tested by measuring the nucleic acid content of control and 6-MP-inhibited bacteria.

Nucleic Acid Content of 6-MP-Inhibited E. coli—*E. coli* cells were grown in glucose-salts media in the presence and absence of 6-MP. After 1.5 hours of growth the cells from each culture were harvested, washed, and extracted with TCA, ethanol, and 10 per cent NaCl as described. The

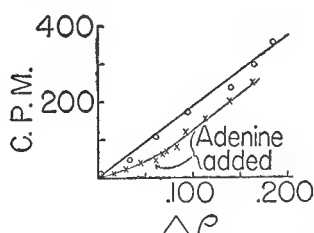


FIG. 5. Adenine reversal of 6-MP-induced inhibition of uracil- C^{14} uptake in *E. coli*. O, control; X, 6-MP-inhibited. Adenine added at arrow.

optical density readings of the salt extracts at $260\text{ m}\mu$ were determined on bacterial samples before and after growth in the presence of 6-MP. Similar determinations were carried out for the control culture. The nucleic acid contents were then computed for the same extent of growth by dividing the increase in absorption at $260\text{ m}\mu$ by the increase in growth measured at $650\text{ m}\mu$. It was found that for eight separate experiments the nucleic acid content of cells grown in the presence of 6-MP ranged from 58 to 76 per cent (mean = 65 per cent) of that determined for untreated bacteria. It was concluded that 6-MP at $10\text{ }\gamma$ per ml. decreased the nucleic acid content of *E. coli*.

As a test of this conclusion, identical inocula of bacteria were grown to the same extent in the presence and absence of 6-MP in cultures which contained a mixture of randomly labeled C^{14} -fructose and $P^{32}O_4^{3-}$. The nucleic acids were extracted with 10 per cent NaCl as described above, and the ultraviolet absorption spectra of the extracts were measured. Aliquots were taken for radioactivity determinations with and without an aluminum absorber in order to measure both the total radioactivity and that from the

P^{32} alone. It is evident from the results in Table I that the bacteria grown in the presence of 10 γ per ml. of 6-MP contained approximately one-half as much nucleic acid, phosphorus, and carbon plus phosphorus as did cells grown in the absence of 6-MP. These results confirm the conclusion that 6-MP treatment decreased the nucleic acid content of *E. coli*, but the composition of the polynucleotides seemed unchanged. A similar effect of 6-MP on both bacterial ribonucleic acid and deoxyribonucleic acid has been observed in other investigations (10).

The absorption spectra of NaCl extracts containing approximately 0.25 mg. of nucleic acid per ml. were also examined in the region of 300 to 340 $m\mu$. Since 6-MP exhibits a characteristic absorption peak in this region (1), any increase in absorption at this region might be evidence for incorporation of the 6-MP into nucleic acids. No such indication of absorption due to the drug could be found. The possibility of more than a

TABLE I
Nucleic Acid Content and Specific Radioactivity of 6-MP-Inhibited E. coli

	Newly formed nucleic acid (arbitrary units) (1)	$C^{14} + P^{32}$ (2)	Specific radioactivity (2) \div (1) (3)	P^{32} (4)	Specific radioactivity (4) \div (1)
		<i>c.p.m.</i>		<i>c.p.m.</i>	
Control.....	100	457	4.57	60.6	0.606
6-MP.....	51	243	4.75	29.4	0.575

small amount of the purine analogue being incorporated is thus precluded. The incorporation of the drug into nucleic acids has been proposed as a possible explanation for its mechanism of action (19).

Effect of 6-MP on Utilization of 1-C¹⁴-Acetate—McQuillen and Roberts have shown (20) that 1-C¹⁴-acetate contributes radioactive carbon principally to the proteins and lipides of *E. coli*. Since no effect of 6-MP upon protein synthesis had been observed in the present studies (Figs. 2 and 3) and since 1-C¹⁴-acetate does not efficiently label the nucleic acids, it was thought that the total uptake of 1-C¹⁴-acetate would provide a convenient indicator of the effect of the drug upon lipid synthesis. Accordingly, the uptake of 1-C¹⁴-acetate by control and 6-MP-treated (10 γ per ml.) bacterial cultures was determined by the membrane filter technique. Sufficient carrier acetate was added to minimize isotopic dilution by C¹²-acetate produced from glucose (6). For the same extent of growth 6-MP-inhibited *E. coli* incorporated only about one-third as much C¹⁴ as did control cells. Since the radiocarbon from 1-C¹⁴-acetate is approximately equally distributed between lipid and protein in control cells (20), the suppression

observed was greater than could be accounted for as a result even of complete lack of lipid synthesis. The results suggest that, in spite of the absence of effect of 6-MP on protein synthesis as measured by sulfur uptake (Fig. 3), acetate utilization for protein synthesis was curtailed by 6-MP. In order to test this suggestion and to determine the distribution of carbon among the different chemical fractions in the cell a series of analyses with various C^{14} -labeled compounds was carried out.

Effect of 6-MP on Distribution of Carbon in E. coli—6-MP-treated and untreated cultures of *E. coli* were grown to an equal extent in the presence of 1- C^{14} -acetate, randomly C^{14} -labeled glucose, C^{14} -formate, or 2- C^{14} -uracil.

TABLE II
Effect of 6-Mercaptopurine on Utilization of C^{14} -Labeled Compounds

Fraction	1- C^{14} -Acetate		C^{14} -Glucose		C^{14} -Formate		C^{14} -Uracil	
	0*	10*	0*	10*	0*	10*	0*	10*
	Radioactivity incorporated†							
Total.....	100	30	100	80	100	65	100	45
Cold TCA-soluble.....	5	4	18	15	4	2	6	1.5
Alcohol-soluble.....	53	15	35	30	7	5	1	0
Hot TCA-soluble.....	3	2	52	34	58	26	77	40
Residue.....	37	8			27	28	3	1.5

* Micrograms of 6-MP per ml. added.

† The values are given in per cent of the radioactivity incorporated by the cells in the control culture. Bacteria in the inhibited cultures were grown for a longer time than those in the control cultures in order to provide the same amounts of cells in each case.

At the conclusion of the growth period the cells were harvested, washed, and fractionated by the chemical fractionation procedure described above. Radioactivity of each of the fractions was determined and calculated as per cent of the radioactivity incorporated by the bacteria in the control cultures.

It is evident from the results (Table II) that 6-MP markedly suppressed the incorporation of C^{14} from 1- C^{14} -acetate into both lipides (alcohol-soluble) and proteins (residue).

In the case of C^{14} -glucose, which randomly labels all of the carbon of the cell (6), the major effect of 6-MP was found in the combined hot TCA and residue fractions. This is probably due to the low nucleic acid content of the 6-MP-treated cells since the sulfur incorporation studies (Fig. 3) indicated a normal protein content in bacterial cells exposed to 6-MP. The small effect on the lipid fraction shows that the lipid content is

only slightly decreased, in spite of the suppression of 1-C¹⁴-acetate utilization.

The results for C¹⁴-formate labeling show that the nucleic acid fraction contained most of the C¹⁴ and that this fraction was the one most affected by 6-MP treatment. Incorporation of formate carbon into the cold TCA fraction, which contains small amounts of purine compounds, was apparently also suppressed. Considerable C¹⁴ was found in the protein fraction in this experiment, arising from the incorporation of C¹⁴O₂ produced from the labeled formate (6). The proteins of a similar preparation were hydrolyzed to amino acids, and the hydrolysates were chromatographed on paper. The distribution of C¹⁴ among the amino acids was typical of that for cells grown in the presence of C¹⁴O₂ (21). In addition, non-radioactive Na₂CO₃ added to cultures containing labeled formate suppressed the incorporation of C¹⁴ into protein. These results indicate that amino

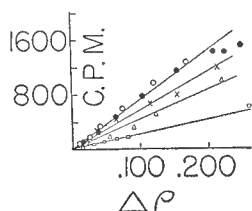


FIG. 6. The effect of 6-MP on 1-C¹⁴-acetate utilization in *E. coli*. ○ = control; ● = 0.5; × = 1; △ = 1.5; □ = 3 γ per ml. of 6-MP.

acid syntheses involving CO₂ utilization were not much affected by 6-MP.

Paper chromatographic analysis of the hydrolyzed nucleic acid in the 2-C¹⁴-uracil experiments showed that the radioactivity was contained entirely in the pyrimidine nucleotide residues. The principal effect of 6-MP was to suppress the incorporation of 2-C¹⁴-uracil into the nucleic acid fraction. Since the cold TCA fraction contains pyrimidine compounds, it was also affected by 6-MP treatment although the amounts of radioactivity present were too low to assure accuracy.

Effect of 6-MP Concentration on Growth, Nucleic Acid Content, and 1-C¹⁴-Acetate Utilization.—Three series of cultures were grown in the presence of 0, 0.5, 1, 1.5, 2, and 5 γ per ml. of 6-MP. The nucleic acid content of one series was estimated by 10 per cent NaCl extraction and spectrophotometry. The distribution of C¹⁴ between the lipides (alcohol-soluble fraction) and proteins (residue) was determined on the second series in which 1-C¹⁴-acetate was used as the labeled compound. The third series was analyzed by means of the membrane filter technique to detect the uptake of 1-C¹⁴-acetate. The results of these analyses are shown in Table III and Fig. 6.

0.5 γ per ml. of 6-MP inhibited the growth of *E. coli* but had no dis-

cernible effect upon the nucleic acid content (Table III). In addition, other experiments showed that this low dosage of drug suppressed the utilization of C^{14} -formate or C^{14} -formaldehyde only in accord with the effect upon growth. It would seem therefore that a decreased nucleic acid content is not the cause of growth inhibition.

The suppression of acetate utilization for both lipid and protein synthesis became progressively greater as the 6-MP concentration was increased. On the other hand, the lipid content as measured by the incorporation of C^{14} -glucose (Table II) or the protein content as measured by the incorporation of $S^{35}O_4^{=}$ (Fig. 3) was not appreciably decreased by 6-MP treatment even at 10 γ per ml. Thus, 6-MP strongly suppressed acetate utilization without much altering the content of lipid or protein.

TABLE III
Relation between Concentration of 6-MP, Growth, Nucleic Acid Content, and 1- C^{14} -Acetate Utilization

	6-MP, γ per ml.					
	0	0.5	1	1.5	2	5
$\rho:\rho_0$ (2 hrs.)	3.9	3.0	2.5	2.5	2.1	2.5
Nucleic acid content*	100	105†	75†	57	50	60
C^{14} into lipides*	100	94	54	41	26	27
“ “ proteins*	100	88	48	47	47	33

* Expressed as per cent of that in the untreated cells for the same extent of growth.

† In other experiments the nucleic acid content at these levels of 6-MP did not differ from that of the control, nor was any specific effect on the uptake of C^{14} -formate observed.

DISCUSSION

The experimental results reported in the present paper show that growth of *E. coli* was markedly inhibited by 6-MP at the lowest level (0.5 γ per ml.) tested. However, no specific effect of this level of the drug on the utilization of various labeled compounds or on protein, lipid, or nucleic acid content was observed. Acetate utilization for protein and lipid synthesis was specifically suppressed, nucleic acid content was decreased, and the utilization of formate was specifically suppressed only at 6-MP levels of 1 γ per ml. or more. No decrease in protein or lipid content was observed even at the highest level tested (10 γ per ml.). It is clear, therefore, that the several metabolic properties examined differ in sensitivity to the drug. In order of decreasing sensitivity they fall in the following sequence: growth, acetate utilization, formate utilization and nucleic acid content, protein and lipid content.

6-MP treatment of *E. coli* specifically suppressed the utilization of exogenous acetate carbon for the synthesis of protein and lipid without also decreasing the protein or lipid content. It is evident, therefore, that one site of 6-MP action involves the mechanisms which control the flow of acetate carbon for synthesis. McQuillen and Roberts (20) have shown, however, that the pathways for the flow of acetate carbon into lipid and protein are largely independent. Since 6-MP affects both pathways, it may be inferred that its influence is exerted on the early stages of acetate carbon utilization. The ready reversibility of the inhibitory effects of 6-MP by purines suggests that the analogue antagonizes the synthesis or utilization of a purine-containing compound. Coenzyme A, adenosine triphosphate, and diphosphopyridine nucleotide might be involved specifically, since they take part in the early stages of acetate utilization and also contain purine residues in their structures. Coenzyme A in addition contains a sulfhydryl group which could conceivably be inactivated by coupling with 6-MP. It is suggested, therefore, that 6-MP exerts its effects on *E. coli* by interfering with the function and synthesis of purine-containing coenzymes. The reversal of 6-MP-induced inhibition of mitosis in sarcoma 180 (22), as well as lipogenesis in embryo skin fibroblasts (23) by coenzyme A, and the inhibition of CoA-mediated acetylation by the drug in normal and tumor tissue (24) lend support to this hypothesis. The demonstration of 6-MP inhibition of diphosphopyridine nucleotide synthesis and breakdown is further corroboration (25).

Since the effect of 6-MP on growth inhibition occurred at the lowest concentration of the drug, it is likely that the effects on nucleic acid synthesis and acetate utilization are secondary. It is very probable, however, that a comparable effect of the drug on one or more specific cofactors may influence the growth rate, even though such responses may be of a magnitude outside the range of the present techniques.

SUMMARY

6-Mercaptopurine (6-MP) inhibits the growth of *Escherichia coli* B. Growth inhibition is reversed by adding purines to cultures of the bacteria. It may also be reversed by subculture of the cells in 6-mercaptopurine-free media or by prolonged incubation of the bacteria in low concentrations of the drug. The capacity of the microorganisms to form the inducible enzyme, β -galactosidase, is unimpaired. Protein synthesis as measured by the incorporation of S^{35} from labeled sulfate is identical in inhibited and control cultures for the same extent of growth. The utilization of acetate for protein and lipid syntheses is strongly suppressed. Nucleic acid synthesis, as measured spectrophotometrically or by the incorporation of radioactivity from labeled glucose, formate, uracil, or phos-

phate in cultures treated with 10 γ per ml. of 6-MP, is almost half that found in control cultures for the same amount of growth. At low levels of 6-MP a decrease in growth rate is produced, although no effect on acetate utilization, formate uptake, or nucleic acid content is observed. It is suggested that 6-MP exerts its effects by interfering with the function and synthesis of purine-containing cofactors.

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IV.B.2 Remplacement total de la méthionine par la sélénométhionine dans les protéines d'*Escherichia coli*

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Washington, D. C.

Un mutant d'*Escherichia coli* incapable de synthétiser la méthionine peut croître exponentiellement et synthétiser des protéines enzymatiquement actives en présence de sélénométhionine et en l'absence totale de méthionine.

R. Munier et G. N. Cohen ⁽¹⁾, ⁽²⁾ ont vu que la para-fluorophénylalanine peut être incorporée à la place de la phénylalanine et de la tyrosine dans les protéines d'*Escherichia coli*. Lorsque la proportion d'analogue n'est pas trop élevée (environ 50 % de remplacement), les protéines synthétisées comprennent encore certains enzymes actifs (β -galactosidase, par exemple). Au contraire, lorsque la teneur en *p*-fluorophénylalanine est maximum, et que toute la phénylalanine et toute la tyrosine sont remplacées, les bactéries ne synthétisent plus de β -galactosidase.

Nous décrivons ici une situation où un analogue structural de la méthionine, la sélénométhionine, remplace intégralement l'acide aminé naturel correspondant. Les protéines formées au cours de la croissance normale observée sont, dans ce cas, fonctionnelles.

Nous avons utilisé le mutant d'*E. coli* ML 304 *d*, exigeant la méthionine pour sa croissance. Ce mutant croît exponentiellement et peut être repiqué indéfiniment sur milieu synthétique additionné de sélénométhionine à la place de méthionine, en présence de sulfate pour assurer la synthèse des autres métabolites soufrés (cystéine, glutathion, vitamines).

Le taux de croissance est réduit par rapport à celui obtenu avec la méthionine (temps de division augmenté de 30 %). Nous avons vérifié régulièrement que le mutant employé était stable; en effet, après 70 générations sur sélénométhionine, nous avons constaté que les bactéries étaient toujours incapables de se développer sur un milieu minimum; d'autre part, nous avons cultivé le mutant sur sélénométhionine en présence de sulfate radioactif, nous avons hydrolysé les protéines et chromatographié l'hydrolysât et n'avons pas trouvé de méthionine radioactive, alors que la cystéine obtenue est hautement radioactive. En outre, la co-chromatographie de méthionine radioactive sans entraîneur et de sélénométhionine (en quan-

tité suffisante pour donner une forte réaction positive à la ninhydrine) nous a montré que la sélénométhionine de synthèse que nous avons employée (provenant du Docteur Alex Shrift, de l'Université de Pennsylvanie) est exempte de toute trace de méthionine.

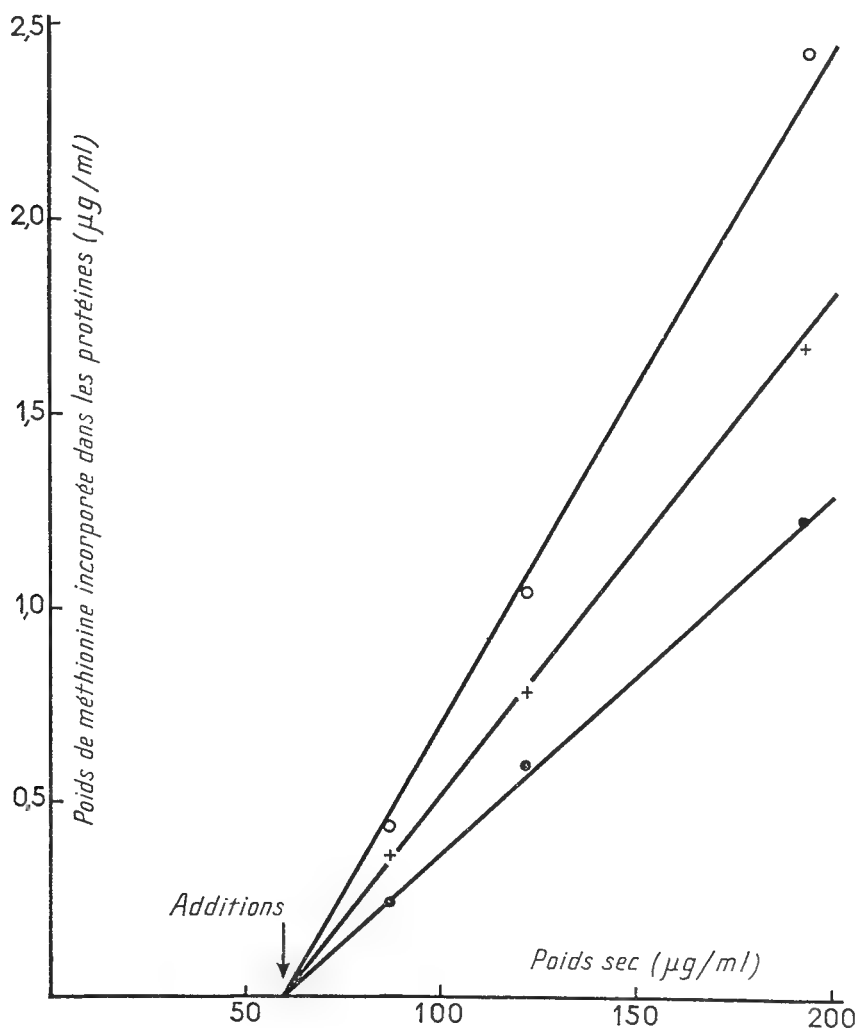


Fig. 1. — Réduction de l'incorporation de la méthionine radioactive par la sélénométhionine (Mutant ML 304 d).

Abscisses : poids secs de la culture au cours de la croissance.

Ordonnées : poids de méthionine incorporée au cours de la croissance.

○ culture témoin; DL-méthionine 5.10^{-5} M.

× Idem + DL-sélénométhionine 5.10^{-5} M.

● Idem + DL-sélénométhionine 10^{-4} M.

DL-méthionine radioactive : 312 000 impulsions-minute/micromole.

Si l'on cultive le mutant en présence d'un mélange de méthionine radioactive (^{35}S) et de sélénométhionine, le taux de croissance est le même qu'en présence de méthionine seule; cependant, on observe une réduction

de 26 % de l'incorporation de la méthionine pour un rapport moléculaire analogue sélénié/méthionine = 1 et de 48 % pour un rapport double (fig. 1).

Nous avons étudié la synthèse induite de la β -galactosidase au cours de la croissance du mutant sur méthionine, sur sélénométhionine ou sur des mélanges en proportions variables de ces deux substances. Si l'on appelle 100 le taux différentiel de synthèse sur méthionine seule, les taux

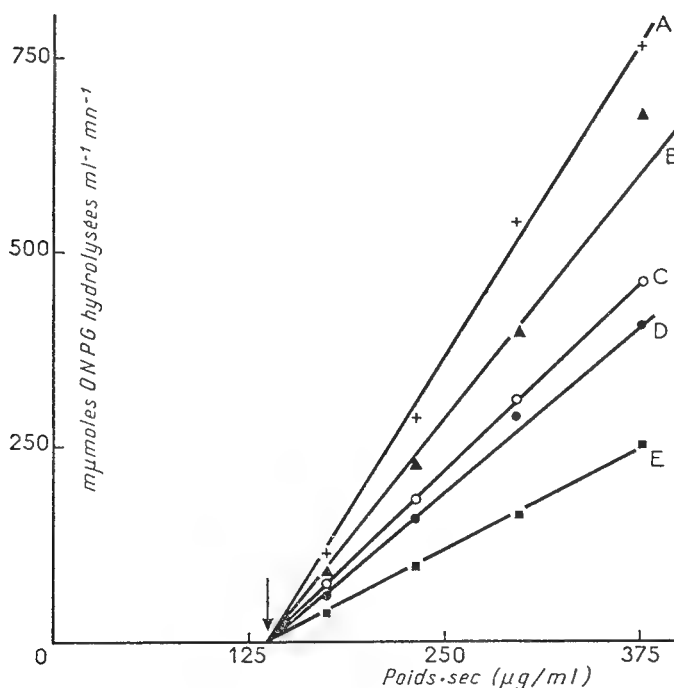


Fig. 2. — Synthèse de la β -galactosidase par le mutant ML 304 *d* en présence de méthionine, de sélénométhionine et de mélanges de ces deux substances en proportions variables.

Abscisses : poids secs de la culture au cours de la croissance.

Ordonnées : Unités d'activité β -galactosidasique (²).

La flèche indique l'addition de l'inducteur de la β -galactosidase qui était le thiométhyl- β -D-galactoside 10^{-3} M.

A : DL-méthionine $5 \cdot 10^{-5}$ M; B : Idem + DL-sélénométhionine $5 \cdot 10^{-5}$ M; C : Idem + DL-sélénométhionine $2,5 \cdot 10^{-4}$ M; D : Idem + DL-sélénométhionine $5 \cdot 10^{-4}$ M; DL-sélénométhionine 10^{-4} M.

différentiels sont respectivement de 77 pour un rapport moléculaire sélénométhionine/méthionine = 1, de 59 pour un rapport de 5, de 50 pour un rapport de 10 et de 35 pour une croissance sur sélénométhionine pure (fig. 2).

Ce dernier résultat peut être attribué soit à la synthèse d'une β -galactosidase dont l'activité spécifique est modifiée, soit à une diminution de la synthèse d'enzyme, soit enfin à une combinaison de ces possibilités. La solution de ce point particulier ne pourra être donnée qu'après isolement de la sélénio- β -galactosidase pure. Quoiqu'il en soit, le présent travail

démontre qu'*E. coli* peut synthétiser des protéines enzymatiquement actives dont un aminoacide est remplacé *totalelement* par un analogue non naturel.

(¹) *Biochim. Biophys. Acta*, **21**, 1956, p. 592.

(²) En préparation.

(³) J. MONOD, G. COHEN-BAZIRE et M. COHN, *Biochim. Biophys. Acta*, **7**, 1951, p. 585.

IV.B.3 Biosynthesis by Escherichia coli of Active Altered Proteins Containing Selenium instead of Sulfur*

(Reprinted, by permission, from Biochimica et Biophysica Acta, vol. 26, pp. 252-261, 1957.) (Received May 6, 1957.)

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INTRODUCTION

SPIEGELMAN¹ has recently raised the question of the microheterogeneity of proteins due to small variations in structures arising at the level of the synthesizing system. The tentative conclusion was reached that "this question of microheterogeneity at the level of a protein-forming site is still an open question".

Attempts to introduce variations in the amino acid composition of protein have been made by culturing organisms in the presence of structural analogs of amino acids²⁻⁴. Molecular analogs of amino acids are incorporated into proteins of growing *Escherichia coli*. For example, 47% of the tyrosine and 23% of the phenylalanine can be replaced by *p*-fluorophenylalanine in the newly formed proteins of wild-type *E. coli*. Furthermore, it has been shown by using a phenylalanine-requiring mutant that all of a normal amino acid can be replaced by this analog⁵. In the former case, active β -galactosidase is synthesized; in the second case, however, the proteins formed do not show such activity. Instead of exponential growth, "linear growth"⁶ is obtained. Under these conditions, since one or more enzymes are not synthesized in an active form⁵, they remain at the same value at which they were at the time the analog was added and become rate limiting.

It was interesting to learn whether an amino acid analog could be found that allowed exponential growth and active enzyme synthesis in the complete absence of the naturally occurring corresponding amino acid.

Such a case has been found. Exponential growth was obtained with the selenium analog of methionine, selenomethionine, using a methionine-requiring mutant of *E. coli*. In this case, sulfate was added in order to insure the needs of the organism for non-methionine sulfur compounds—glutathione, cysteine, vitamins, etc. We have also investigated the capacity of the mutant to utilize selenite instead of sulfate, in the presence of methionine, *i.e.*, its capacity to utilize selenite for the synthesis of the selenium analogs of glutathione and cysteine. The synthesis of β -galactosidase has been followed under all these conditions.

* This work has been supported by grants from the Rockefeller Foundation of New York, the Jane Coffin Childs Memorial Fund for Medical Research, and the Commissariat à l'Energie Atomique.

** Permanent address: Dept. of Terrestrial Magnetism, Carnegie Institution of Washington, Washington, D.C. This work was done during a visit of DEAN B. COWIE as a guest of the Institut Pasteur.

PROCEDURES

Radioactive isotopes. $^{75}\text{SeO}_3\text{H}_2$ in HCl solution (original radioactivity of 937 mc/g), ^{35}S -sulfate and ^{35}S -DL-methionine (original radioactivity of 5 mc/mmmole) have been used.

Chemical compounds. DL-selenomethionine was synthesized⁷ and kindly given to us by Dr. ALEX SHRIFT, Department of Botany, University of Pennsylvania. Thiomethyl- β -D-galactoside (TMG), thioisopropyl- β -D-galactoside (IPTG), inducers of β -galactosidase synthesis, and o-nitrophenyl- β -D-galactoside (ONPG), a substrate of this enzyme, were synthesized in this laboratory.

Growth. Bacteria were grown, unless otherwise stated, on medium 63³ with maltose (2 g/l) as the carbon source, and shaken at 37° C. They were cultured overnight and allowed to resume exponential growth before any experiment was started. When the methionine-requiring mutant was used, the overnight culture was carried out with $5 \cdot 10^{-4} M$ DL-methionine. Growth was followed at 6,000 Å, in the "spectrophotometre Jean et Constant" (Prolabo, Paris) and a known conversion factor was applied to convert the readings into bacterial dry weights.

Sulfur-free medium. Described in ref.⁸.

β -Galactosidase induction and assays. TMG or IPTG was added to an inducible strain one min after the other additions at concentrations stated for each experiment. The standard assays of β -galactosidase were made on toluenized suspensions of bacteria in $2.7 \cdot 10^{-3} M$ ONPG and $5 \cdot 10^{-2} M$ sodium phosphate buffer (pH 7.0), according to MONOD, COHEN-BAZIRE AND COHN⁹.

Chromatography. Protein hydrolyzates⁸ were analyzed by two-dimensional chromatography (1st dimension: BuOH/HCOOH/H₂O, 70/10/20; 2nd dimension: phenol/NH₄OH/H₂O, 80/0.3/20) and detected with ninhydrin and by radioautography on Kodak radiography films (Kodirex: 30 \times 40 cm; emulsion on both sides).

Radioactivity measurements. Aliquot samples were spread on aluminum planchets after removal of trichloroacetic acid (TCA) and counted with an end-window counter with a mica window of 1.5 mg/cm². The counter was used with a conventional 100-scaler.

Strains used. *Escherichia coli* ML30, inducible for β -galactosidase and its mutant, requiring methionine, ML 304d were used. This mutant is the one used by COHN, COHEN AND MONOD¹⁰ to estimate methionine; it has an absolute and stable block between homocysteine and methionine.

RESULTS

PART I

Growth of mutant ML 304d with methionine and selenomethionine

Fig. 1 shows the growth of mutant ML 304d with the addition of either methionine or selenomethionine to the medium 63. The growth rate is decreased with selenomethionine (division time 73 min compared to 55 min), but growth is exponential. A control culture without methionine or selenomethionine did not grow.

Competition between methionine and selenomethionine for protein synthesis

Cultures of the methionine-requiring mutant were grown in the presence of $5 \cdot 10^{-5} M$ radioactive methionine, $5 \cdot 10^{-5} M$ selenomethionine or with $5 \cdot 10^{-5} M$ radioactive methionine plus $10^{-4} M$ selenomethionine. Fig. 2 shows that growth rates of the three cultures were identical. Throughout the experiment, samples were taken and after fractionation of the cells, the radioactive methionine content of the residual protein fraction was determined. Fig. 3 shows that the methionine content of the cells was reduced by 28% for a 1:1 ratio of selenomethionine to methionine, and by 50% for a 2:1 ratio of selenomethionine to methionine. These results show that selenomethionine competes with methionine for protein synthesis. The essential controls of this experiment were:

(a) Chromatography of the synthetic selenomethionine (at quantities large enough to give a strong ninhydrin reaction) mixed with carrier-free ^{35}S -methionine showed that the seleno-analog is free of any trace of methionine.

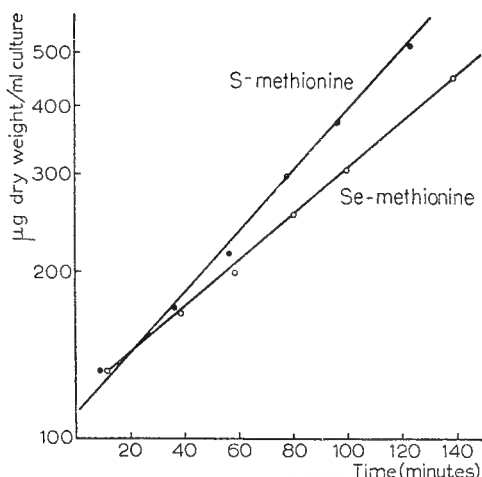


Fig. 1. Exponential growth of ML 304d. Washed 5 times with saline, resuspended in medium 63. At time $t = 0$, DL-methionine (●) or DL-selenomethionine (○) added at a concentration of $10^{-4} M$.

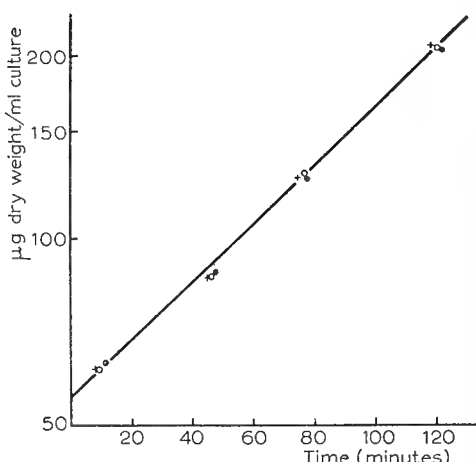


Fig. 2. Growth rates for the mutant ML 304d grown in the presence of methionine or with methionine plus selenomethionine (the growth rate begins to be slightly depressed when the ratio selenomethionine/methionine reaches 10). + DL-methionine $5 \cdot 10^{-5} M$; ○ DL-methionine $5 \cdot 10^{-5} M$ + DL-selenomethionine $10^{-4} M$.

(b) The mutant used is stable. It had been verified previously that it cannot convert homocysteine to methionine in contrast to the wild type^{10 11}. It does not incorporate radiosulfur from sulfate into methionine. Similar results had been obtained by COWIE AND BOLTON¹² in another methionine-requiring strain of *E. coli*. The protein synthesized by the ML 304d mutant when grown with radiosulfate and selenomethionine was hydrolyzed and chromatographed; the methionine region showed no trace of radioactivity, while the cysteine region was highly radioactive.

Continuous growth of the methionine-requiring mutant on selenomethionine

Starting with an inoculum of 10^7 cells, the mutant ML 304d has been grown with $5 \cdot 10^{-5} M$ selenomethionine and transferred daily on this medium for a length of time corresponding to more than 100 generations. During this period, tests for reversions were made by plating the culture on complete agar and on minimal synthetic agar; no reversion was ever detected. However, while growth was exponential and the cells looked normal to microscopic examination and normal colonies were obtained on complete agar or on minimal agar supplemented with methionine, no colonies could be obtained on minimal agar supplemented with $10^{-4} M$ selenomethionine. No explanation for the different behavior of the mutant in the liquid medium and on the agar plates can be given at this time.

Synthesis of β -galactosidase by the mutant growing on methionine and selenomethionine

Studies have been carried out on the differential rates of synthesis* of β -galactosidase¹³ (Δ enzyme/ Δ mass) by growing the mutant ML 304d on methionine, on selenomethionine and on various mixtures of both compounds. Fig. 4 shows that if the

* The differential rate of synthesis of a given component is defined as the ratio of the increase of that component to the increase of the bacterial mass.

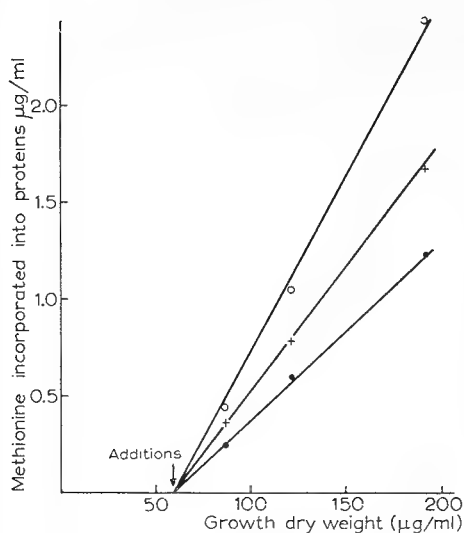


Fig. 3. Reduction of the incorporation of radioactive methionine by selenomethionine. Mutant ML 304d. Abscissa: dry weight of the culture during growth. Ordinate: methionine incorporated into the residual proteins during growth. W: control culture; DL-methionine ($5 \cdot 10^{-5} M$). +: same as control plus DL-selenomethionine ($5 \cdot 10^{-5} M$). ●: same as control plus DL-selenomethionine ($10^{-4} M$).

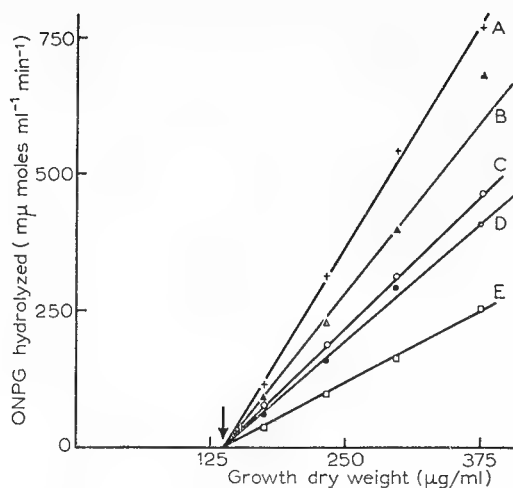


Fig. 4. Synthesis of β -galactosidase by mutant ML 304d in presence of methionine, selenomethionine and various mixtures of the two metabolites. Abscissa: Dry weight of the cultures during growth. Ordinate: $m\mu$ moles ONPG hydrolyzed $ml^{-1} \times min^{-1}$, at $28^\circ C$. The arrow indicates the addition of TMG ($10^{-3} M$). A: DL-methionine ($5 \cdot 10^{-5} M$). B: same as A plus DL-selenomethionine ($5 \cdot 10^{-5} M$). C: same as A plus DL-selenomethionine ($2.5 \cdot 10^{-4} M$). D: same as A plus DL-selenomethionine ($5 \cdot 10^{-4} M$). E: DL-selenomethionine ($10^{-4} M$).

differential rate on methionine is arbitrarily called 100, the differential rates of synthesis are 77 for a molecular ratio selenomethionine/methionine of 1; 59 for a ratio of 5; 50 for a ratio of 10; and 35 when the culture is made on selenomethionine alone.

This result could be attributed to (a) the synthesis of an enzyme with a modified affinity for the substrate; (b) a reduction of the total enzyme synthesized; or (c) the synthesis of a β -galactosidase of a lower specific molecular activity. Direct determinations of the affinities of ONPG for the active extracts have shown no significant difference between the affinity constants of the enzymes obtained from bacteria grown on either methionine or its seleno-counterpart. The last possibility could be tested only by measuring the turnover numbers of both normal β -galactosidase and seleno- β -galactosidase. Whatever the answer to this question will be, the present work shows unequivocally that *E. coli* can grow and synthesize active proteins under conditions where an amino acid is totally replaced by a synthetic unnatural analog.

PART II

Since it has been established that 100% of the methionine in *E. coli* proteins can be replaced by selenomethionine in exponentially growing cells, it seemed desirable to ascertain whether selenium could entirely replace sulfur for this organism.

In order to resolve this problem, it was necessary to recognize that exponentially growing *E. coli* contain large reservoirs of internal sulfur, which can be used for

residual protein synthesis during sulfur starvation. It has been shown⁸ that some growth is invariably observed when exponentially growing cells are transferred to a medium in which no sulfur is intentionally added. The two-phase growth curve similar to that shown in Fig. 5 is characteristic of the growth in sulfur-deficient media. From these data, the growth phases can be represented by the equations*:

$$N = N_0 e^{k_1 t} \quad \text{and} \quad N = N_1 e^{k_2 t}$$

where N_0 equals the quantity of cells per unit volume at time of inoculation and N_1 the quantity of cells at the end of the first exponential growth period; k_1 is the same coefficient as found in experiments with adequate sulfur; and k_2 appears to be roughly

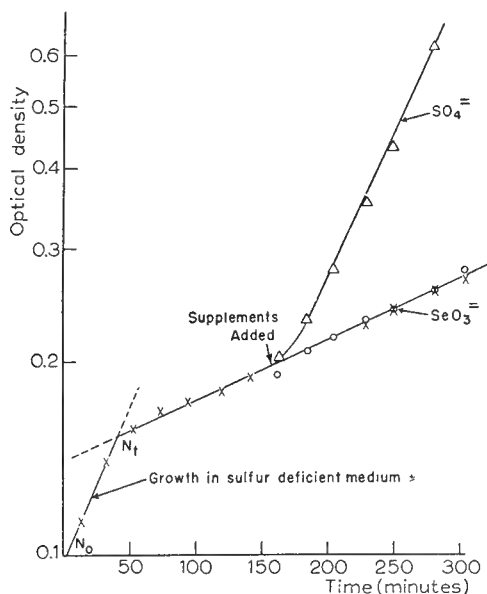


Fig. 5. Wild-type ML 30 was grown exponentially on medium 63, washed 4 times with saline, and transferred to two flasks containing the S-deficient medium. Growth was followed until all the glutathione was used up. During the alcohol-soluble protein utilization, $1.25 \mu\text{g S/ml}$ were added to one flask (Δ) and $3 \mu\text{g Se/ml}$ were added to the other (\circ).

$1/10 k_1^{**}$. During the initial phase, the cells use any trace quantities of S in the medium and also their internal content of glutathione-sulfur, which serves as a reservoir¹⁴. When the glutathione is exhausted, the growth rate drops abruptly. This change marks the beginning of the second growth phase. During this phase, the cells utilize a less readily available reservoir of sulfur, the alcohol-soluble proteins⁸.

Incorporation of selenium from radioselenite into the proteins of E. coli

Thus, in order to investigate whether selenium could *entirely* replace sulfur for cysteine and methionine synthesis, it was necessary to exhaust first the large glutathione sulfur reservoir before the addition of selenite.

* Since the second growth phase cannot be studied over a sufficiently long period, it can be equally well represented by the expression $N_1(1 + k_2 t)$, so that it is presently not known whether this phase is exponential or linear.

** When methionine is present, k_2 is roughly $1/5 k_1$.

The experiment of Fig. 5 demonstrates that the growth rate of cells which have exhausted their glutathione is unaffected by the presence of selenite. In a control culture, upon the addition of sulfate optimal growth rate was immediately obtained. These results suggest that selenium cannot entirely replace sulfur in *E. coli*. However, since it has been shown that methionine can be replaced by selenomethionine, it seemed desirable to determine whether conditions existed where selenium could be utilized for the synthesis of selenogluthathione or selenocysteine. In order to exclude any influence of selenite upon methionine synthesis, exogenous methionine was routinely added when wild-type cells were used, or when the experiments were performed with the methionine-requiring mutant ML 304d.

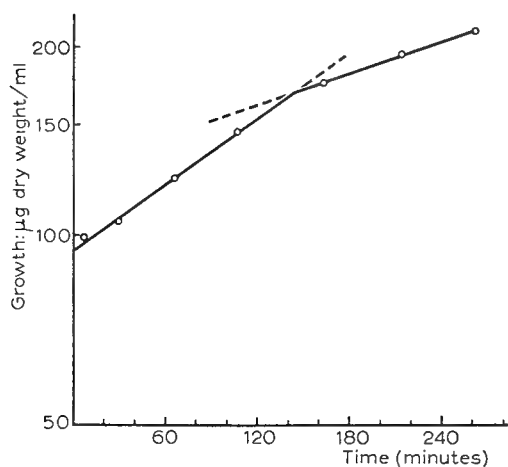


Fig. 6a. Mutant ML 304d. Growth with selenite (no sulfur added). Cells were harvested during exponential growth, washed 5 times with saline, and resuspended in S-deficient medium containing 10^{-4} M DL-methionine and radiosenite ($3.9 \mu\text{g Se/ml}$; specific activity: $7,440 \text{ counts/minute}/\mu\text{g Se}$).

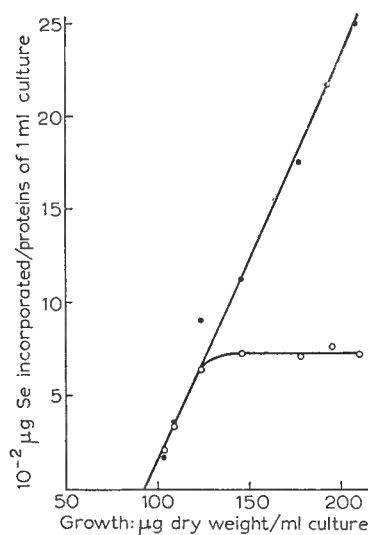


Fig. 6b. Mutant ML 304d. Incorporation of radiosenite during the two growth phases of Fig. 6a. Note that the incorporation of selenium in the residual proteins is directly proportional to the new cell mass, even though there is the characteristic two-phase growth curve observed during sulfur starvation. ●: residual proteins. ○: alcohol-soluble proteins.

When endogenous glutathione sulfur is available, the typical diphasic growth curve (Fig. 6a) is observed and radiosenite is incorporated into the cellular proteins (Fig. 6b). The incorporation in the residual protein fraction is directly proportional to the new cell mass (Fig. 6b). Upon chromatographic examination of the hydrolyzates of this protein fraction, it is found that the radioactivity is contained in a region where cysteine is normally found. Thus the presence of limiting quantities of sulfate or of internal glutathione permit the synthesis and incorporation of a selenium-containing material having chromatographic properties similar to those of cysteine.

Although radioactivity is found in the proteins, no radioactivity, hence no selenium, is found in the glutathione fraction (cold TCA-soluble fraction), showing that selenogluthathione is not made from selenite.

Since these results demonstrate a significant difference between the utilization

of selenomethionine and that of selenite, kinetic investigations were carried out in order to relate the two phenomena.

Competition between selenite and sulfate for protein synthesis

The following experiment was performed, using the wild-type ML 30, with methionine present in the medium. Four cultures were made, one containing radiosulfate ($2 \mu\text{g S/ml}$); the second radiosulfate ($2 \mu\text{g S/ml}$) plus nonradioactive selenite ($3.9 \mu\text{g Se/ml}$); the third radioselenite ($3.9 \mu\text{g Se/ml}$) plus nonradioactive sulfate ($2 \mu\text{g S/ml}$); the last culture contained radioselenite ($3.9 \mu\text{g Se/ml}$). Samples were taken at intervals during the exponential growth phase corresponding to the utilization of the internal glutathione and of the limiting quantities of exogenous sulfate. The results shown in Fig. 7 demonstrate that sulfate and selenite reduce the incorporation of each other.

Furthermore, the incorporation of S and Se is directly proportional to the quantity of new cellular mass. The quantities of S and Se incorporated in the residual proteins of the cells have been calculated from the slopes of the straight lines shown in Fig. 7. These results are given in Table I. Since the growth rates were identical in the three first cultures, it can be concluded that a fraction of the cysteine sulfur can be replaced by selenium. If one expresses the selenium in sulfur equivalents, it is seen that 10% of the cysteine sulfur would be replaced by selenium, under the conditions of the experiment ($\text{Se/S} = 0.78$).

Analysis of the data (Fig. 8, curve B) shows that β -galactosidase is made at the normal rate (curve C) only during the first part of the utilization of the internal glutathione sulfur. During the latter part of the glutathione sulfur utilization and during the entire alcohol-soluble protein degradation period, none of this enzyme was formed. This suggests that the alcohol-soluble proteins cannot provide sulfur in an adequate quantity (or form) for the *de novo* synthesis of β -galactosidase.

When selenite is added to cells containing both their glutathione and alcohol-soluble reservoirs, the synthesis of β -galactosidase occurs only during the first part of glutathione sulfur utilization (Fig. 8, curve A). There are two significant differences that occur upon the addition of selenite:

- (a) More total growth occurs during the first growth phase, indicating that the

TABLE I
COMPETITION BETWEEN SULFUR AND SELENIUM FOR PROTEIN SYNTHESIS*

Flask	Contents	S in μg	Se in μg	S equivalent of Se**	Total "sulfur"
		per g dry weight residual protein			
A	Radiosulfate (2 μg S/ml)	957			957
B	Radiosulfate (2 μg S/ml) + Selenite (3.9 μg Se/ml)	847			
C	Radioselenite (3.9 μg Se/ml) + Sulfate (2 μg S/ml)		215	87.1	934.1

* *E. coli*, wild type ML 30. DL-methionine was added to all cultures at the concentration of $10^{-4}M$. Data obtained from the slopes of Fig. 7.

** S equivalent was obtained by multiplying the Se value by 32/79, ratio of the corresponding at. wt. The data were obtained from the slopes of the curves reproduced in Fig. 7.

selenium is acting as a sparing agent for the utilization of sulfur, confirming the results of Fig. 7.

(b) The differential rate of synthesis of enzyme, as well as the total amount are reduced.

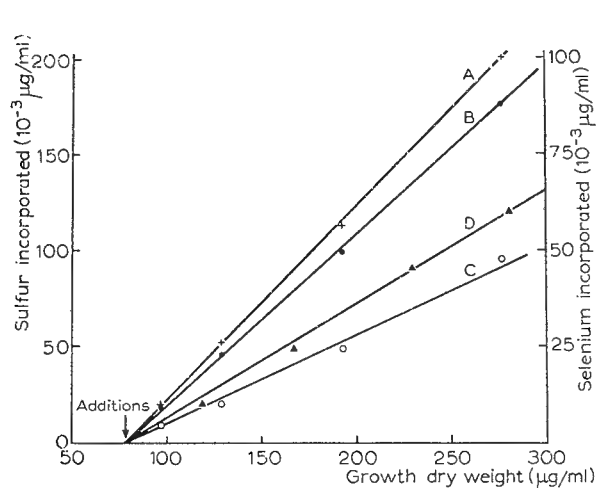


Fig. 7. Sulfur and selenium incorporation into the residual proteins of wild-type ML 30. DL-methionine ($10^{-4}M$) was present in all flasks. Culture A: radiosulfate ($2 \mu g S/ml$). Culture B: same as A, plus selenite ($3.9 \mu g Se/ml$). Culture C: radioselenite ($3.9 \mu g Se/ml$) plus sulfate ($2 \mu g S/ml$). Culture D: radioselenite ($3.9 \mu g Se/ml$) ^{35}S (37,500 counts/min/ $\mu g S$) ^{75}Se (7,440 counts/min/ $\mu g Se$).

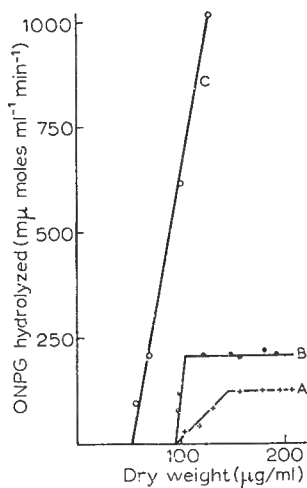


Fig. 8. Induced synthesis of β -galactosidase in mutant ML 304d during sulfur starvation. Inability of selenium to replace sulfur for optimal synthesis. An exponential culture was washed 5 times with saline and resuspended in S-deficient medium. Additions were made as indicated one minute after the addition of the inducer (IPTB $3 \cdot 10^{-3}M$). Methionine ($10^{-4}M$) present in all flasks. Selenite: $3.9 \mu g Se/ml$. A = no sulfate added; selenite added. B = no sulfate added. C = adequate sulfate.

During these experiments, the incorporation of radioselenium from selenite was followed in both the residual and alcohol-soluble protein fractions. As shown in Figs. 6a and 6b, the incorporation of selenium into residual protein is directly proportional to the new cell mass, even though there is the characteristic two-phase growth curve observed during sulfur starvation. During this period, the alcohol-soluble proteins also incorporate radioselenium proportionally to new cell mass during the first part of the glutathione utilization, but cease to incorporate selenium at about the same time as enzyme synthesis ceases. It is significant that the quantity of selenium incorporated in the alcohol-soluble proteins remains constant, whereas it is known that, during this period, sulfur of the alcohol-soluble proteins is transferred to residual proteins and growth is observed. It is also important to recognize that although no selenium is lost or incorporated from the alcohol-soluble fraction, selenium continues to be incorporated into the residual protein fraction of the cells, indicating both that residual protein synthesis continues in the cell and that the selenium must be derived from exogenous sources.

DISCUSSION

It has been known for a long time that selenate toxicity is associated with the formation of selenium-containing organic materials. HORN AND JONES¹⁵ reported the isolation from *Astragalus pectinatus* of a crystalline amino acid complex containing both selenium and sulfur: its tentative structural formula is indicative of a mixture of cystathionine and its selenium analog. However, selenocysteine or selenomethionine have never been isolated pure from plant, animal or bacterial organisms having grown in the presence of selenate or selenite.

FELS AND CHELDELIN¹⁶⁻¹⁸ in a series of studies on selenate toxicity have found that:

(a) In yeast, selenate toxicity is reversed by L-methionine and by no other compound tested, including cysteine. This effect is also found in rats¹⁶. The toxicity is believed to be due to a competitive inhibition between sulfate and selenate resulting in the blocking of methionine synthesis. As methionine can be used as the sole source of sulfur in yeast¹⁷, it is easily understandable that addition of this amino acid suppresses selenate toxicity.

(b) In *E. coli*, cysteine and, to some extent, glutathione, but not methionine nullify the selenate growth inhibition¹⁸. These results are consistent with our findings that selenogluthathione is not made from selenite and that selenocysteine is synthesized to a limited extent only. Selenomethionine is not toxic and, in fact, it can serve as the sole "methionine" source for *E. coli*. It is possible that selenocysteine is not adequate for the synthesis of active proteins because (a) the inability of forming Se-S or Se-Se bridges, or (b) the incompatibility between proteins containing such linkages and a normal catalytic activity. It is also possible that the responsible enzymes of *E. coli* are incapable of transforming selenocysteine to selenomethionine. A more definite answer to the questions raised by this work must await the availability of selenocysteine in order to test its availability as a "cysteine" source and as a selenomethionine precursor.

SHRIFT^{19,7} has studied the action of selenate and selenomethionine on the growth of *Chlorella vulgaris*, but the results were obscured by the fact that this organism synthesizes methionine from sulfate and also because methionine can serve, as in yeast, as the sole sulfur source. His results, however, strengthen the view that organic analogs of sulfur metabolites are made from selenate. SHRIFT also makes the hypothesis that these organic analogs might be metabolized. Both views are supported by the work reported here on selenite metabolism and selenomethionine utilization as the sole source of "methionine" for the growth of a methionine-requiring mutant.

The fact that exponential growth is observed when selenomethionine completely replaces the methionine demonstrates the synthesis of all the essential enzymes. When other structural analogs of amino acids were studied (*p*-fluorophenylalanine, β -2-thienylalanine, norleucine^{3,5}), linear growth always occurred, accompanied by the cessation of synthesis of one or more active essential enzymes. In each case the analogs were incorporated into the proteins replacing structurally related natural amino acids. These results demonstrate that the amino acid composition of proteins may be influenced by environmental changes.

Also relevant to the problem of microheterogeneity are the results (COHEN, in the press) of the effect of L-valine on the growth of *E. coli* (K-12). This naturally occurring

amino acid causes linear growth at small concentrations. This growth is accompanied by the cessation of the synthesis of several active enzyme systems and by drastic changes in the amino acid composition of the proteins formed. Since the incorporation of amino acid analogs into protein results, either in the synthesis of fully active enzymes or in the suppression of synthesis of some enzymes, these analogs become powerful tools for the study of: (a) the specificity of the protein-forming mechanism and (b) the variation of enzyme activity and affinity that occurs with increasing numbers of incorporated analog molecules.

SUMMARY

1. Selenomethionine can completely replace methionine for the normal exponential growth of a methionine-requiring mutant of *E. coli*. β -Galactosidase is formed under these conditions.

2. Selenium cannot entirely replace sulfur for the growth of *E. coli*. However, in the presence of the glutathione sulfur reservoir or with traces of sulfate, selenium partially replaces sulfur. Hydrolyzates of the residual protein fraction having incorporated radioselenium contain a radio-active material with chromatographic properties similar to those of cysteine. The incorporation of selenium is proportional to the increase in bacterial mass. No selenogluthathione is formed. Selenium is also incorporated into the alcohol-soluble proteins, but cannot be transferred from it to the residual proteins during sulfur starvation.

Active β -galactosidase cannot be formed at the expense of the alcohol-soluble proteins whether selenium is present or absent.

3. The results reported here and elsewhere demonstrate that the amino acid composition of the proteins may be influenced by the presence of exogenous amino acids or their analogs, and suggest that some variation in the amino acid composition of a given protein may be the rule rather than the exception.

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IV.B.4 Amino Acid Analog Incorporation into Bacterial Proteins

(Reprinted, by permission, from Biochimica et Biophysica Acta, vol. 34, pp. 39-46, 1959.) (Received October 6, 1958.)

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SUMMARY

The amino acid analogs norleucine and para-fluorophenylalanine are shown to be incorporated into the proteins of *E. coli*. Analysis of proteins by an ion-exchange column showed that the proteins formed in the presence of the analogs are not radically different molecular species but are physicochemically similar to the proteins normally synthesized. The substitution of norleucine for methionine in the bacterial proteins was shown to occur in the same proportions in all of the "protein classes" resolved by the ion-exchange column.

INTRODUCTION

Considerable quantities of certain amino acid analogs may be incorporated into the proteins of *Escherichia coli*¹⁻⁶. The analogs substitute for corresponding naturally occurring amino acids and cause various biological effects. In general, cellular growth becomes linear with time and specific enzymic activities may be lost, depressed, or remain unaffected. Such effects depend upon the degree and kind of substitution produced. Since the degree and kind of substitution can be controlled, the use of analogs provides a method for the quantitative examination of the relationship between altered molecular structure and enzymic activity. Evidence can also be adduced concerning susceptibility of bacterial protein types to analog substitution.

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PROCEDURES

Wild type *E. coli* ML 30 and a methionine-requiring mutant, ML 304d, were used in these experiments. The cells were cultured in vigorously aerated C medium*** with maltose (1.0 g/l) as the carbon source. Thiomethyl β -*d*-galactoside (TMG) $5 \cdot 10^{-4}$ M was used to induce the synthesis of β -galactosidase.

DL-[14 C]phenylalanine (10^{-4} M, $6 \cdot 10^5$ counts/min/ μ M), DL-[14 C]*p*-fluorophenylalanine ($5 \cdot 10^{-4}$ M, $4.3 \cdot 10^4$ counts/min/ μ M); L-[35 S]methionine (10^{-4} M, $6 \cdot 10^5$ counts/min/ μ M), DL-[14 C]norleucine ($2 \cdot 10^{-2}$ M) were the radioactive tracers used. The radioactive DL-[3- 14 C]phenylalanine and DL-[3- 14 C]*p*-fluorophenylalanine were synthesized by Dr. PICHAT of the Commissariat à l'Energie Atomique, France. L-[35 S]-methionine was obtained from the Abbott Laboratories, Chicago, Illinois; the DL-[14 C]-norleucine was obtained from the Volk Radiochemical Company, Chicago, Illinois.

Cell extracts

After growth in the presence of either the [14 C]phenylalanine or [14 C]*p*-fluorophenylalanine, the cells were harvested and washed twice with 0.02 M sodium phosphate buffer, pH 7.0, and resuspended in 10 ml of the same buffer at a bacterial concentration of 15 mg dry wt./ml. The cells grown in medium containing [35 S]-methionine or [14 C]norleucine were washed in TSM buffer* pH 7.6 and resuspended in 10 ml of the same buffer. In each case the cells were ruptured by extrusion through a small orifice under pressure (approximately 16,000 lb./sq. in.) in a modified** French pressure cell⁷. The extruded material was centrifuged for 10 min at 13,000 rev./min in a Servall centrifuge to remove whole cells, cell walls, and other large fragments. An aliquot of the opalescent supernatant (approximately 8 ml) was placed on a N-diethylaminoethyl-cellulose (DEAE) ion-exchange column⁸. The remainder was used to determine the total radioactivity, total β -galactosidase, or other enzyme activities and for chemical fractionation⁹. Only a small fraction of the radioactivity and a negligible fraction of the enzyme activity were present in the large fragment pellets.

The amount of β -galactosidase was determined by the rate of hydrolysis of *M*/375 ortho-nitrophenyl β -*d*-galactoside in 0.05 M sodium phosphate buffer, pH 7, at room temperature by toluenized preparations of the whole cells prior to rupture, by the extract prior to fractionation through the column, and by aliquots of the individual tubes collected from the DEAE column. Phosphoglucomutase¹⁰ and glucose-6-phosphate dehydrogenase¹¹ were also estimated in those aliquots.

DEAE-cellulose ion-exchange column

The DEAE-cellulose ion-exchange material was prepared as described by PETERSON AND SOBER⁸. The particular batch used throughout these experiments contained 1.0 mequiv. of nitrogen per gram dry weight. The exchanger was stored as a

* C Medium: 2 g NH₄Cl, 6 g Na₂HPO₄, 3 g KH₂PO₄, 3 g NaCl, 0.01 g Mg as MgCl₂, 0.026 g S as Na₂SO₄, 100 ml 10% maltose, and 900 ml distilled H₂O.

** TSM buffer: 0.01 M tris succinate, 0.005 M Mg as magnesium acetate.

*** The blind end of the pressure cell cylinder was bored out and a removable pressure seal inserted in order to facilitate loading, cleaning, and assembly. The operation of the cell was as described previously⁷.

moist slurry at room temperature in 0.1 *M* NaCl at pH 10. Immediately before use, a portion was washed in the centrifuge with 0.02 *M* phosphate buffer or TSM until the suspending fluid was neutral. Columns were prepared by settling several aliquots of the neutral slurry into a chromatographic tube (1 cm diameter) until the height of the column under 10 lb. pressure reached 20 cm. Such a column contained 2.5 g dry exchanger. The supernatant fluid of the pressure cell extract obtained from approximately 120 mg dry weight of bacteria was adsorbed to the column under pressure (10 lb./sq. in.). The column was eluted with a linear salt gradient (0.0 *M* to 0.8 *M* NaCl in 0.02 *M* phosphate buffer or TSM pH 7.6) supplied by the device of BOCK AND LING¹². Eluates were collected in 20 drop (approx. 1 ml) fractions at a flow rate provided by an air pressure of 3–5 lb./sq. in. (about 0.15 ml/min). 100–200 tubes were collected at room temperature and stored at 4° until appropriate analysis could be made. Columns freshly prepared from the stock suspension were used for each experiment. Radioactivity, protein content¹³, and enzyme activities were determined on aliquots from each of the fractions collected for each experiment.

RESULTS

The demonstration that amino acid analogs could be incorporated into bacterial proteins immediately raised many questions concerning the nature of the proteins produced. Investigations were carried out to determine whether the analogs are contained in radically different molecular species or in proteins similar to those normally synthesized. These investigations required (a) the use of an analog that would substitute for only one naturally occurring amino acid; and (b) a quantitative method for analyzing bacterial proteins.

The analog, *norleucine* substitutes for methionine in the proteins of *E. coli*. A reduction of about 38 % of the protein methionine is obtained when the methionine requiring mutant (ML 304d) is grown in C-medium containing DL-norleucine ($2 \cdot 10^{-2}$ *M*) and L-^[35S]methionine (10^{-4} *M*). This mutant was chosen in order to eliminate competitive reactions involving sulfur compounds other than methionine or the methionine analog. The separation of bacterial proteins into chromatographically resolvable "protein classes" was achieved through the use of the DEAE cellulose ion exchange column. Fig. 1 shows the elution pattern of an extract of *E. coli* grown for many generations in C-medium containing ^[35S]methionine. Thiomethyl β-*D*-galactoside was added to induce the synthesis of β-galactosidase.

The bacterial extract was prepared from washed cells, ruptured by extrusion through a small orifice under pressure, and the extruded material centrifuged to remove whole cells and large cellular fragments. The opalescent supernatant was then used for the column analysis. Evident in this elution diagram are a number of well resolved regions showing a close correlation between the protein pattern (measured by the Folin procedure¹³) and the pattern of distribution of the radiomethionine. Two dimensional paper chromatograms of hydrolysates of an aliquot of the bacterial extract showed that the incorporated radioactivity was contained solely as methionine.

In Fig. 2 are shown the specific radioactivities of the individual fractions. The specific radioactivity is defined as the ratio of the quantity of radioactivity to the amount of protein synthesized after the addition of the radioactive amino acid.

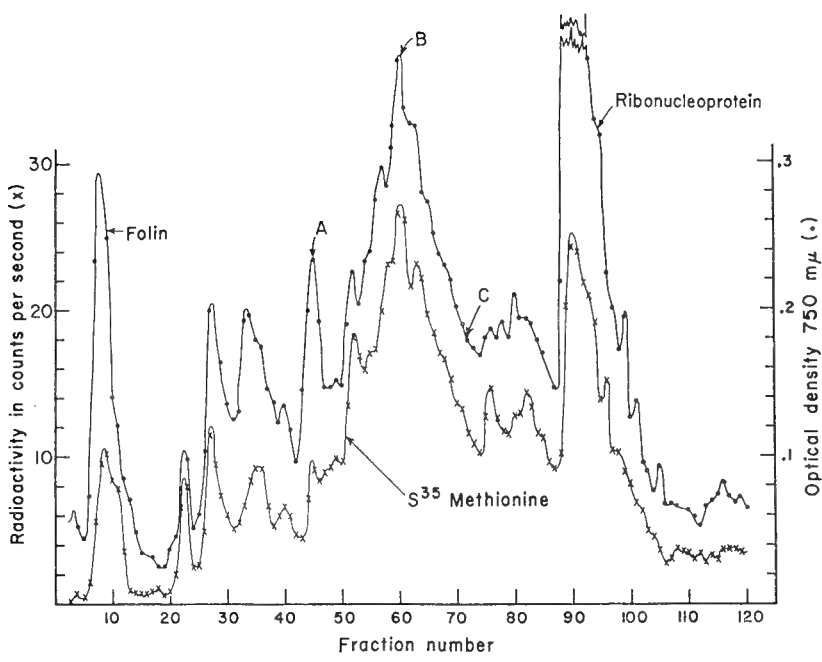


Fig. 1. Elution pattern of bacterial extract of *E. coli* obtained with ion-exchange column. Mutant cells (ML 304d) grown in C-medium containing L-[³⁵S]methionine (10^{-3} M); thiomethyl β -D-galactoside ($5 \cdot 10^{-4}$ M) and maltose.

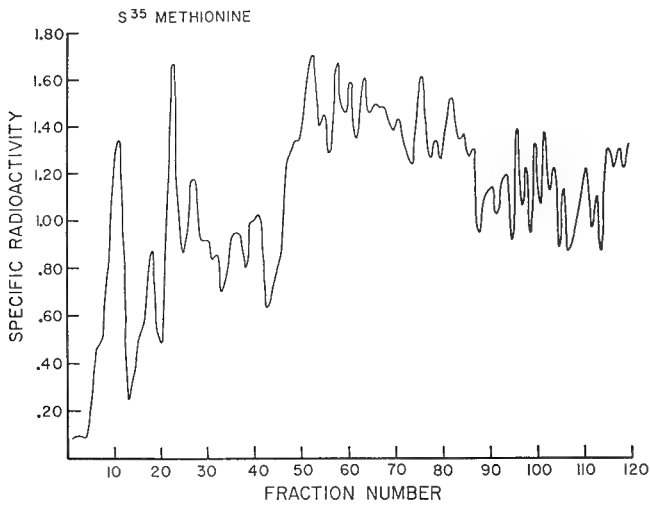


Fig. 2. Specific radioactivity of eluted column fractions (in arbitrary units). Data represent the ratio of radioactivity per fraction to the quantity of protein newly synthesized after the addition of the labeled methionine to the culture.

Fig. 3 shows the degree of resolution among the eluted proteins. Superimposed on the elution diagram are the locations of 3 enzyme activities; β -galactosidase (LAC), phosphoglucomutase (MUT), and glucose-6-phosphate dehydrogenase (ZW). Each enzyme activity can be correlated with an obvious protein peak. Other proteins

having similar charge properties are, of course, contained in each region; nevertheless the partitioning of bacterial proteins into "protein classes" is apparent.

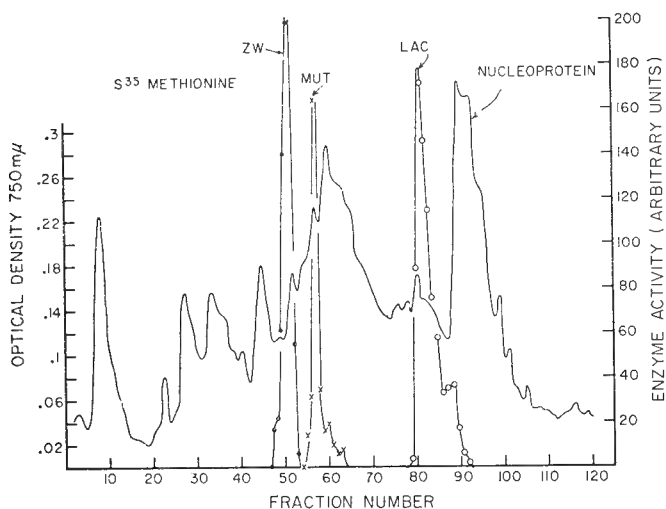


Fig. 3. Location of enzyme activities along elution diagram. Glucose-6-phosphate dehydrogenase (ZW); phosphoglucomutase (MUT); β -galactosidase (LAC).

Column analysis of bacterial extracts of cells grown in C medium containing DL-norleucine ($2 \cdot 10^{-2} M$) and L- $[^{35}S]$ methionine ($10^{-4} M$) gave elution patterns similar to Fig. 1. A significant difference was a *uniform reduction* in the specific radioactivities of these bacterial proteins compared to those of the control experiment (Fig. 2). The existence of certain markers (peaks, valleys, enzymes, etc.) along the elution diagram allows a quantitative comparison, marker for marker, among several column runs. Fig. 4 shows the specific radioactivities of seven well marked and separated regions obtained with the norleucine grown cells. These are compared to the same regions in the control experiment, where the specific radioactivity of each region was arbitrarily chosen to equal 100.

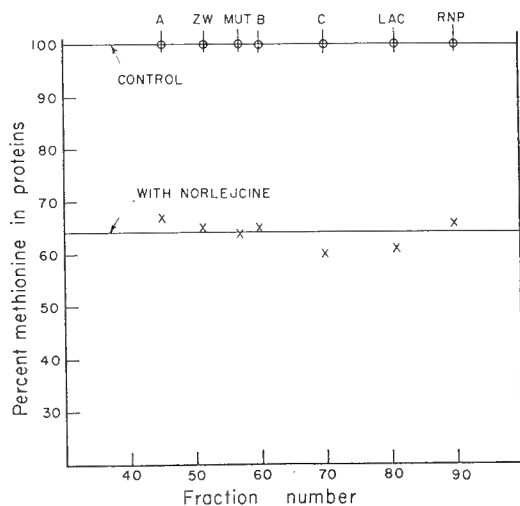


Fig. 4. Comparison of specific radioactivities of definite regions along elution diagrams obtained from *E. coli* ML 304d cells grown in C-medium containing L- $[^{35}S]$ methionine ($10^{-3} M$) (control) and from cells grown in C-medium containing L- $[^{35}S]$ methionine ($10^{-4} M$) plus DL-norleucine ($2 \cdot 10^{-2} M$). Linear growth was obtained in the latter culture and the cells were harvested for analysis after more than a doubling of bacterial mass.

The regions compared in Fig. 4 were: 2 well resolved and isolated protein peaks A and B; the ribonucleoprotein peak (Fig. 1); and the 3 peaks of enzyme activity (LAC, MUT, and ZW) (Fig. 3) easily measurable in both experiments, and region C (Fig. 1). Each point in Fig. 4 represents the arithmetical mean of the specific radioactivity of the maximum peak sample and the two samples immediately preceding and the two following this peak.

Fig. 5 shows an elution pattern obtained from cells grown in DL- ^{14}C norleucine ($2 \cdot 10^{-2} M$) and nonradioactive L-methionine ($10^{-4} M$). In this experiment there was a 43 % substitution of norleucine for methionine in the bacterial proteins. Radioautograms of two dimensional paper chromatograms of hydrolysates of the bacterial extract showed one radioactive, ninhydrin-positive spot having the same R_F as found with the labeled norleucine used in this experiment.

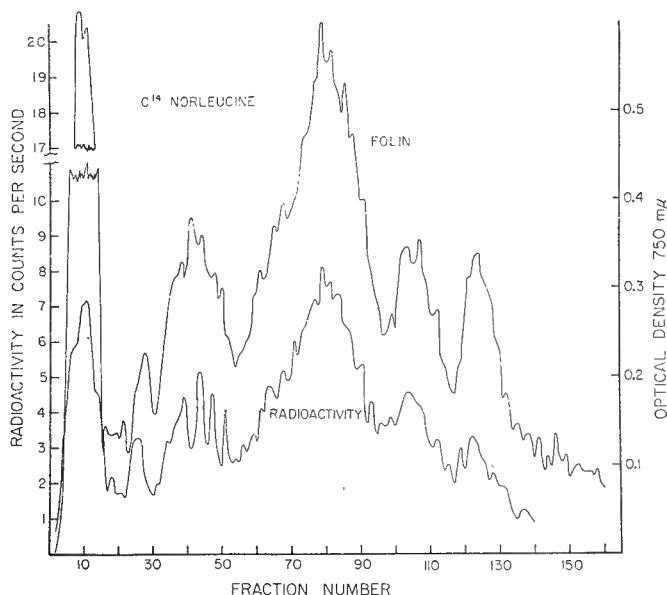


Fig. 5. Elution pattern of bacterial extract of DL- ^{14}C norleucine grown cells. Mutant *E. coli* cells (ML 304d) grown for more than two doublings of bacterial mass in C-medium containing labeled norleucine ($2 \cdot 10^{-2} M$) and L- ^{35}S methionine ($10^{-4} M$).

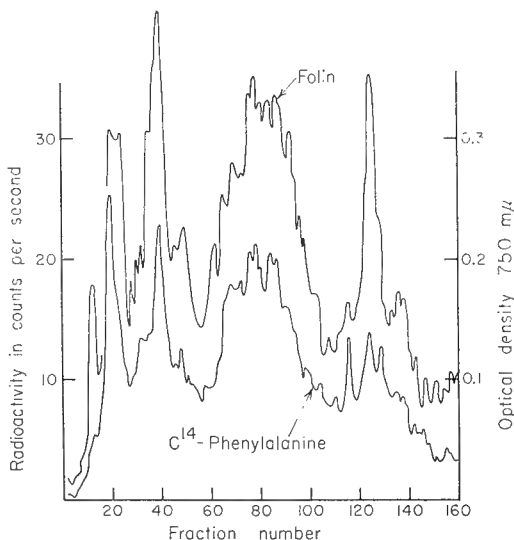
There is a great deal of similarity in the elution diagrams obtained from the ^{35}S methionine (Fig. 1) and the ^{14}C norleucine labeled cells (Fig. 5). One significant difference, however, occurs in the first major peak of the elution diagrams. In these early fractions of eluted material are contained the non-protein amino acids (or analogs) concentrated by the cell from the environment. The quantity of "free amino acids" depends upon their external concentrations and in these experiments the ratio of DL- ^{14}C norleucine to L- ^{35}S methionine in the media was 100 to 1. Chemical fractionation of the eluted fractions showed that TCA-soluble material ("free amino acids") was mainly contained in the first 20 samples and dropped rapidly to a few per cent by the 35th sample, remaining low for the rest of the elution process. It has also been noted that the quantity of material contained in the ribonucleoprotein region varies from one column run to another and if the cells are ruptured in media

containing phosphate buffer or in buffer containing an inadequate magnesium concentration this ribonucleoprotein is not seen at all.

DISCUSSION

The above results demonstrate that most of the proteins formed in the presence of the analog are not radically different molecular species, but are physicochemically similar to the proteins normally synthesized. The similarity of the elution diagrams obtained with the labeled methionine and norleucine (Figs. 1 and 5) also eliminates the suggestion that only certain proteins are susceptible to analog substitution. Indeed Fig. 4 demonstrates that the analog is incorporated into all of the proteins examined *in the same proportion*. Each methionine incorporation site thus seems to have an equal probability of analog substitution. The formation of a large quantity of uncompleted proteins caused by the joining of the analog by a peptide bond to one of its neighboring amino acids, but not to the other does not seem to be a probable event. Should such unfinished molecules be present, they would markedly alter the elution patterns obtained after the analog is incorporated.

Fig. 6. Elution pattern of bacterial extract obtained with DEAE ion-exchange column. Wild type *E. coli*, ML 30 grown in C-medium containing DL-[3-¹⁴C]phenylalanine (10^{-4} M) plus DL-parafluorophenylalanine ($5 \cdot 10^{-3}$ M). Linear growth was obtained and the cells were harvested for analysis after more than a doubling of cellular mass.



These conclusions are strengthened by data obtained in expts. using other amino acid analogs. Fig. 6 shows the elution diagram obtained from wild type *E. coli* (ML 30) grown in C-medium containing DL-[3-¹⁴C]phenylalanine (10^{-4} M) and DL-*p*-fluorophenylalanine ($5 \cdot 10^{-3}$ M). At these concentrations there is approximately a 50 % substitution of the analog for protein phenylalanine, and linear growth occurs. The elution diagram obtained (Fig. 6) appears quite similar to that obtained from normal cells (Fig. 1). There is no evidence of different types of "protein classes" being formed as a result of analog substitution.

Fig. 7 demonstrates, within the limits of resolution of the column that *p*-fluorophenylalanine is incorporated into all the bacterial proteins. This elution diagram was obtained from wild type *E. coli* grown in C-medium containing DL-[3-¹⁴C]*p*-fluorophenylalanine ($5 \cdot 10^{-3}$ M).

The use of another amino acid analog gave results which in every respect confirm

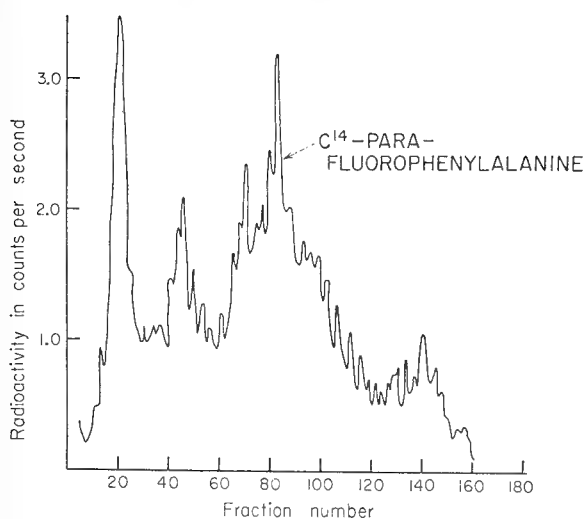


Fig. 7. Elution pattern of bacterial extract obtained with DEAE ion exchange column. Wild type *E. coli*, ML 30 grown in C-medium containing DL-[3-¹⁴C] *p*-fluorophenylalanine ($5 \cdot 10^{-3} M$). Linear growth was obtained and the cells were harvested for analysis after more than a doubling of cellular mass.

and augment the conclusions cited above. Selenomethionine *completely* substitutes for the methionine of the bacterial protein⁴. With this *uniform* replacement exponential growth was observed and the induction and synthesis of active β -galactosidase demonstrated. The constitutive enzymes, essential for exponential growth were obviously present in active forms. Under these conditions there can be little doubt that active altered proteins are synthesized, having biological as well as physicochemical properties similar to those of the normal cell.

The use of amino acid analogs other than selenomethionine has always resulted in linear growth of the cells whenever analog substitution in the protein was evident. Thus, it might be argued that at least one growth-rate-limiting enzyme was unusually susceptible to analog substitution, and the enzyme molecules are synthesized at a reduced rate, if they are synthesized at all. An alternative hypothesis would be that during analog incorporation the protein molecules continue to be synthesized but these would be proteins with reduced capacity for enzymic function. This elimination (or depression) of enzyme activity would depend upon the degree and kind of substitution involved and on the amino acid composition of the sites of enzyme action in the protein molecule. Some evidence supporting the latter hypothesis has accumulated and the investigation of this question is currently under way.

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Comment. Significantly, Yoshida and Tamasaki, publishing in the same issue of Biochimica et Biophysica Acta, reported their findings demonstrating the uniform incorporation of ethionine into normal peptide-bond sequences of a single protein, α amylase. Thus the substitutions of analogs are occurring in the same proportions not only in all the "protein classes" resolved by ion exchange columns but also into different locations of the same protein. Dean B. Cowie.

IV.B.5 The Effect of 5-Fluorouracil on Bacterial Protein and Ribonucleic Acid Synthesis

(Reprinted, by permission, from Biochimica et Biophysica Acta, vol. 49, pp. 98-107, 1961.) (Received November 18, 1960.)

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SUMMARY

1. In the presence of 5-fluorouracil, a large amount of material accumulates that sediments with the smaller ribosomes. Upon removing the analogue, this material is not converted to larger particles.

2. Fluorouracil has no effect on either the differential rate of incorporation of amino acids nor on the relative amino acid composition of logarithmically growing cells.

3. In magnesium starved bacteria, fluorouracil inhibits both ribosome synthesis and protein synthesis. The effect appears to be directly on ribosome metabolism.

4. The correlation of the selective inhibition of protein synthesis and of ribosome metabolism is discussed.

INTRODUCTION

In the previous paper¹, evidence was presented suggesting that FU interfered with the production of phage precursor protein. The fact that the analogue does not affect the synthesis of a number of induced and constitutive enzymes in *E. coli*^{2,3} suggests that the inhibition of phage protein synthesis may be unique. The selective inhibition by the analogue could be due to only certain types of proteins being affected or all proteins but only under certain conditions. Since FU is known to be extensively incorporated into the RNA of *E. coli*^{2,4}, it was conceivable that there was an alteration in nucleic acid metabolism that could account for the selective inhibition.

In the present paper, the effect of FU on RNA metabolism, especially ribosome synthesis is studied. In addition, protein synthesis as measured by incorporation of radioactive precursors and by enzyme induction is examined in normal bacteria and in those in a metabolic state analogous to phage infected cells. The implications of a correlation between inhibition of the synthesis of proteins and of a certain ribosome fraction is discussed.

Abbreviations: FU, 5-fluorouracil; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; TCA, trichloroacetic acid, Tris, tris(hydroxymethyl)aminomethane.

E. coli B or ML 30 was grown overnight in C medium⁵ with limiting glucose or maltose to about 1 g wet wt./l. The cells were diluted 2 or 3 fold in C medium and rejuvenated for about 1 h. Actively growing cells were centrifuged, washed and resuspended to about $5 \cdot 10^8$ /ml for experimentation.

For $^{32}\text{PO}_4$ labelling experiments, cells were suspended in C medium with 0.1 the usual phosphate concentration or in Tris buffer medium⁶.

To measure incorporation into cells, samples were pipetted into cold 5 % TCA, and then filtered through millipore filters. Each filter was washed two or three times with TCA, glued into a planchet, dried and counted in a windowless gas flow counter.

Preparation of extracts

To stop further incorporation, cultures were poured onto an equal amount of frozen, crushed C medium⁷. The cells were centrifuged and washed twice with Tris (0.01 M)–succinate (0.004 M)– 10^{-2} M Mg acetate pH 7.4 in the cold. The final concentrated suspension was passed through the fine orifice of a French pressure cell (10,000–15,000 lb/in²). Cell walls and other debris were removed by centrifuging at 40,000 rev./min for 5 min in No. 40 rotor of Spinco model L (40K/5). The ribosome fraction was prepared by centrifuging the supernate in the same rotor for 2–3 h (40K/(120–180p)). The pellet was resuspended in Tris–succinate– 10^{-2} M Mg acetate and spun briefly in the Servall SS1. The supernate was decanted and stored in the deep freeze.

Sucrose gradient fractionation

Sucrose gradients were prepared as previously described⁸. Fifteen drop fractions (about 0.2 ml) were collected from the bottom of the tube. The adsorption at 260 m μ and TCA-precipitable radioactivity were determined for each fraction.

Analytical ultracentrifuge

Crude extracts (spun at 40K/5 min) and the ribosome fractions (about 2 mg/ml) were examined in the Spinco model E with Schlieren optics. Pictures were taken at 2 or 4 min intervals at 50,740 rev./min.

Magnesium starvation

Logarithmically growing cells were harvested, washed twice with C medium lacking magnesium (M) and resuspended in M (at about $2 \cdot 10^8$ /ml) with an excess of glucose. Cells were vigorously aerated in a 37° water bath for at least 14 h. At this time the absorbancy at 650 m μ had increased two to three times. The cells were centrifuged, washed once and resuspended (usually to $(5-6) \cdot 10^8$ /ml) for experimentation.

Alkaline phosphatase

Cells were grown in Tris medium, centrifuged, washed twice and resuspended in Tris medium with 10^{-5} M phosphate added. 1-ml samples were pipetted into tubes in an ice bath. Two or three drops of toluene were added and the tubes incubated at 37° for 20–30 min. At zero time, 2 ml of substrate (Sigma ONPP dissolved in glycine buffer pH 9.1) were added. Absorbancy changes at 400 m μ in the Beckman DU Spectrophotometer were followed until a constant rate had been established. Results are reported as increase at A_{400} /min/ml.

Chemicals

5-fluorouracil was provided by the Hoffman LaRoche Co. $^{35}\text{SO}_4$ and $^{32}\text{PO}_4$ were

purchased from Oak Ridge, [^{14}C]amino acids and nucleic acid precursor from California Biochemical Corporation. [^{14}C]glucose and [^{14}C]fructose (uniformly labelled) were generously provided by the Department of Terrestrial Magnetism, Carnegie Institution of Washington.

Amino acid hydrolysis

Cells grown in [^{14}C]glucose or [^{14}C]fructose were centrifuged, washed three times with cold 5 % TCA, twice with ethanol-ether (3:1) and finally suspended in 5 % TCA and hydrolyzed at 90° for 15 min. The final pellet was carefully drained and an aliquot (about 3 mg of protein) was suspended in 0.6 ml of 6 *N* HCL. The suspensions were placed in a sealed tube and hydrolyzed at 109° for 12–14 h. The hydrolysate was diluted with water and filtered through Whatman No. 1 filter paper. The filtrate was then evacuated to dryness and redissolved in distilled water. This procedure was repeated 5 or 6 times. Separation of the amino acids was achieved by two dimensional chromatography⁵. Chromatograms were radioautographed to locate spots and all counting done directly on the paper with an end window Geiger-Müller tube.

RESULTS

Effect of FU on ribosome synthesis

When a crude extract from logarithmically growing cells is examined in the analytical ultracentrifuge, a number of characteristic, discrete peaks can be seen^{9,10}. In addition to a large slow moving fraction representing soluble proteins, etc., there are 4 or 5 other peaks (nucleoproteins) with sedimentation coefficients of approx. 20, 30, 50, 70 (and sometimes 88 and 100).

On the basis of radioactive labelling experiments, these nucleoprotein peaks have been shown to be metabolically interrelated⁹. The data is consistent with the smaller components being precursors of the 50- and 70-S particles. In addition, these larger ribosomes break down rather frequently so that large molecules are constantly circulating.

Any alteration in this metabolic pattern, therefore, should be reflected in a change in the steady state amounts of these components. In Fig. 1, a Schlieren photograph clearly shows that the relative quantity of material in the smaller peaks has increased

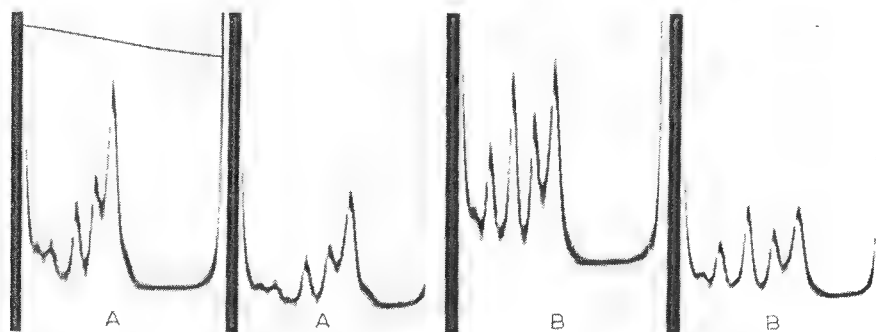


Fig. 1. Schlieren photograph of extract (40K5S) taken at 2-min intervals (first picture taken about 5 min after 50,740 rev./min). Sedimentation from left to right. Bar angle, 40° . Approximate s_{20} values from right to left 85, 70, 50, 30, 20. A, extract-control; B, incubated with fluorouracil, 60 min.

considerably following a 60-min incubation with FU. This alteration is evident even when the ribosome fraction ($40\text{K}120\text{P}$) is examined (Fig. 2).

Either a redistribution of pre-existing components or an accumulation of FU-RNA in the smaller particles (or material having sedimentation properties similar to the smaller ribosomes) could explain this pattern. To distinguish between these two possibilities, cells were incubated with $^{32}\text{PO}_4$ and FU. The ribosome fraction was then sedimented through a sucrose gradient. In Fig. 3, the higher specific activity of the material sedimenting as smaller particles clearly shows that the RNA synthesized in the presence of FU has accumulated here. After a 40-min incubation of control cells, the specific activities of all the ribosomal components would have been equal⁹.



Fig. 2. Schlieren photograph of $40\text{K}120\text{P}$ taken at 2-min intervals, 50,740 rev./min. Bar angle, 35° . Approximate s_{20} values as Fig. 1. A, control; B, incubated with fluorouracil, 45 min.

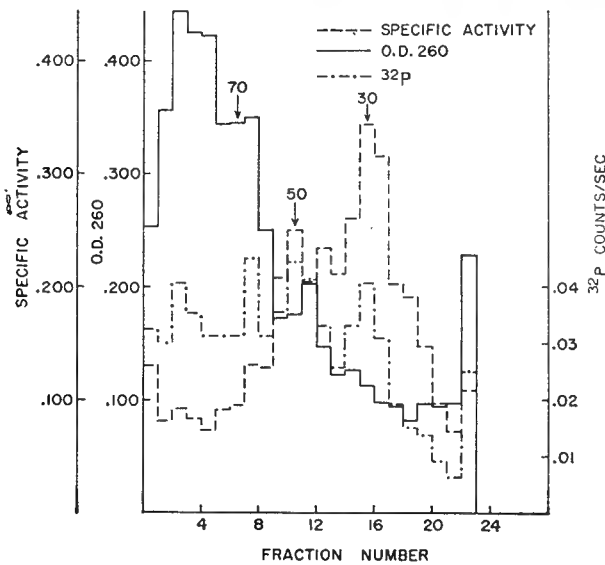


Fig. 3. Sucrose gradient fractionation of $40\text{K}120\text{P}$ isolated from cells incubated for 40 min in the presence of $^{32}\text{PO}_4$ and FU ($20\text{ }\mu\text{g/ml}$). Right hand ordinate (i.e. ^{32}P counts/sec) $\times 10^3$.

Abnormal ribosome synthesis or metabolic block

The accumulation of the RNA immediately suggested two possibilities: (a) the fluorouracil (or a derivative) was directly interfering with the conversion of the smaller components to larger ribosomes. (b) Due to the excessive presence of FU in RNA, "abnormal" ribosomes (or RNA) are formed that cannot be converted to 70-S particles.

By determining whether the accumulated material can be converted to larger ribosomes following removal of FU, these possibilities can be resolved. In the presence of the analogue, the net synthesis of RNA is about 80 % of the control (40 min incubation). This extensive accumulation (especially of material sedimenting with the smaller ribosomes) could have a considerable effect on subsequent synthetic activity. It was, therefore, necessary to examine the synthetic capacity of cells following a 40-min incubation with FU.

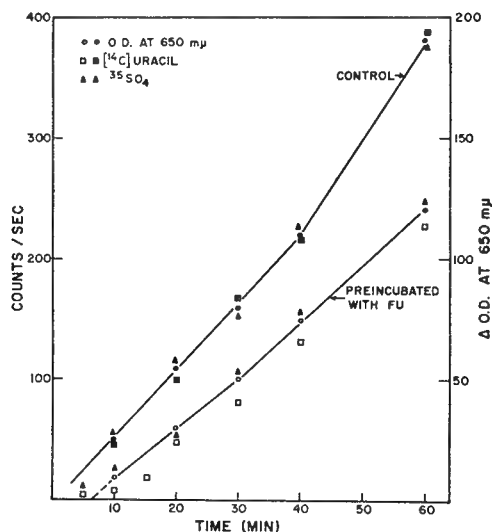


Fig. 4. Changes in absorbancy at 650 mμ and incorporation of $^{35}\text{SO}_4$ and $[2\text{-}^{14}\text{C}]\text{uracil}$ into the TCA precipitable fraction of cells preincubated for 40 min in the presence and absence of FU (20 μg/ml). After washing, both cultures were resuspended in C medium (about $5 \cdot 10^8$ /ml) supplemented with uracil, cytosine and thymidine (30 μg/ml each) plus either $^{35}\text{SO}_4$ or $[^{14}\text{C}]\text{uracil}$ and aerated at 37°.

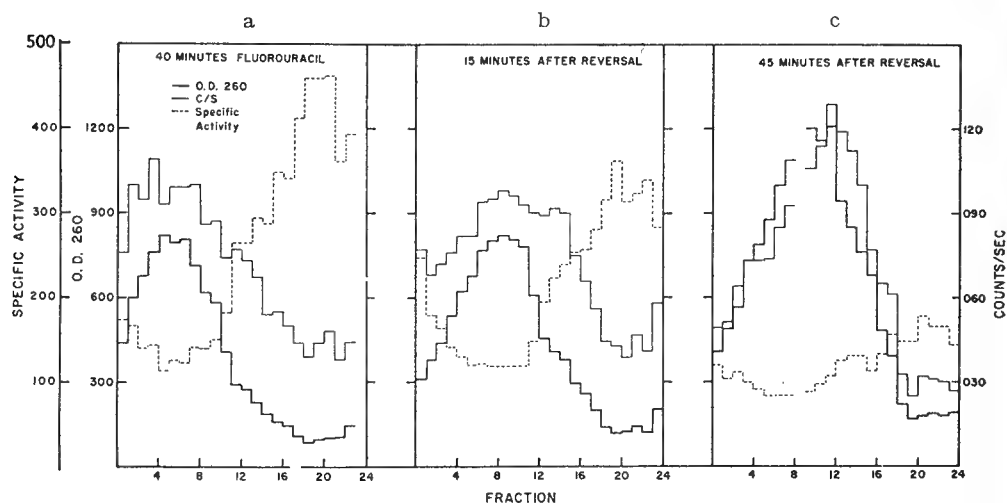


Fig. 5. Sucrose gradient fractionation of 40K180P from cells incubated for 40 min with $^{32}\text{PO}_4$ and FU (20 μg/ml), a. Washed and resuspended to same absorbancy as in a in C medium and incubated for 15 min, b. As in b incubation for 45 min, c. See Fig. 3 for approximate location of ribosomal peaks.

After incubation in the presence of the analogue, the cells were pelleted, washed once with C medium and resuspended to the same absorbancy ($650\text{ m}\mu$ in a Baush and Lomb colorimeter) with excess uracil, cytosine and thymidine. Uptake of $^{35}\text{SO}_4$, $[^{14}\text{C}]\text{uracil}$ and absorbancy changes at $650\text{ m}\mu$ were followed (Fig. 4). Since all cells were viable following resuspension (determined by triplicate plate counts), the lag in RNA and protein synthesis in cells preincubated with FU probably reflects the behavior of the whole population rather than of a certain fraction.

On the basis of persistent labelling experiments (*i.e.* a short pulse followed by a long incubation in nonradioactive medium) it has been concluded that any precursor RNA in the smaller ribosomes should require about 5 min (in C medium, 37°) to equilibrate⁹. Because of the lag in synthetic activity following preincubation with FU, this equilibration time may be extended as much as three fold.

Cells incubated for 40 min with $^{32}\text{PO}_4$ and FU were washed twice with C medium, and resuspended with an excess of uracil, cytosine and thymidine ($40\text{ }\mu\text{g/ml}$ each). Aliquots were taken at 15 and 40 min after resuspension and the ribosome fractions analyzed by sucrose gradient fractionation. The results are summarized in Fig. 5. After 15 min of incubation, the specific activity of the smaller ribosomes is still considerably higher. Even after 40 min, there is no equilibration although by this time, the specific activities have been diluted by the synthesis of nonradioactive RNA. In addition, it is possible that some of the labelled RNA has been lost due to instability of fluorouracil-containing RNA³.

The presence of FU, therefore, apparently results in the accumulation of abnormal ribosomes that cannot be converted to the larger particles.

Protein synthesis in the presence of fluorouracil

If ribosomes have a major role in protein synthesis, then the effect of FU on RNA metabolism should have serious consequences on the capacity of the cell to synthesize protein. In Fig. 6, the differential rate of incorporation of $[^{14}\text{C}]\text{proline}$ in cells incubated in the presence and absence of FU is shown. Similar curves were obtained with $[^{14}\text{C}]\text{leucine}$ or $^{35}\text{SO}_4$. For a period of about one generation, there appears to be little if any effect on amino acid incorporation.

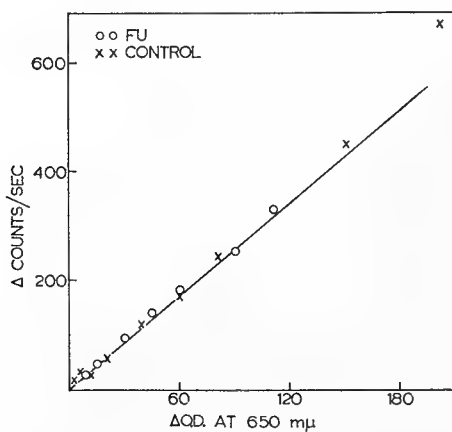


Fig. 6. Differential rate of incorporation of $[^{14}\text{C}]\text{proline}$ into the TCA-insoluble fraction of cells incubated in the presence or absence of FU ($20\text{ }\mu\text{g/ml}$).

In addition, an examination of the relative amino acid composition of cells incubated with or without FU supports this conclusion (Table I). While there are some fluctuations in different analyses in the relative quantity of a few amino acids, the deviations appear to be the same in both the control and experimental analyses.

TABLE I
RELATIVE AMINO ACID CARBON CONTENT OF PROTEIN SYNTHESIZED IN
THE PRESENCE OR ABSENCE OF 5-FLUOROURACIL

Amino acid	Incubated with FU						Control*	
Valine	0.76**	0.75**		0.71***	0.62***	0.74**	0.71***	0.67***
Arginine	0.91	0.88	0.88***	0.97	0.96	0.93	0.94	0.88
Lysine	1.14	1.23	1.00	1.11	1.21	1.03	1.10	1.15
Threonine	0.54	0.64	0.55	0.68	0.52	0.60	0.61	0.56
Glycine	0.35	0.45	0.47	0.45	0.37		0.47	0.41
Serine	0.40	0.49	0.47	0.51	0.46		0.51	0.51
Glutamic acid	1.36	1.32	1.41	1.39	1.52	1.36	1.34	1.55
Aspartic acid	0.95	0.92	1.14	1.10	1.08	0.99	1.05	1.12
Proline	0.58	0.64		0.61		0.54	0.56	0.60
Tyrosine	0.32	0.72	0.53	0.48	0.36	0.70		0.57
Phenylalanine	0.69	0.81				0.76		0.87
Isoleucine } Leucine }	1.71	1.64				1.60		1.71
Histidine					0.26			0.21
Methionine SO ₂					0.20			0.25

* Results reported as counts/min relative to alanine. Some values omitted due to poor resolution of amino acids.

** [¹⁴C]fructose-labelled cells.

*** [¹⁴C]glucose-labelled cells.

Protein and RNA synthesis in magnesium-starved bacteria

On the basis of the previous results, it appears that the inhibition of phage protein synthesis by FU represents a rather special case. It is possible, however, to produce a physiological state of bacteria that in some respects is analogous to a phage-infected cell. By extensive magnesium starvation, the ribosome content of *E. coli* can be reduced to 5–10% of normal^{9,11}. Upon re-addition of magnesium, all cells are viable, and after a short lag, begin ribosome synthesis at an exponential rate. None of the pre-existing ribosomal material is directly utilized, but appears to be slowly degraded to nucleotides¹¹. The analogy to a phage-infected cell would be that in both cases, it is presumably only the DNA that has the capacity to "direct" the synthesis of specific proteins.

If normal ribosome metabolism is required in such cells before protein synthesis can proceed, then FU should have a much more pronounced effect in magnesium starved bacteria than it appears to have in logarithmically growing cells. As the Schlieren diagram in Fig. 7 shows, there is an extensive inhibition by FU of ribosome synthesis in magnesium starved bacteria, especially of the faster moving 70-S component. Even after about 5 h when the control pattern has been completely restored to normal (and the cells first divide) there is very little additional increase in the size of the peaks in extracts prepared from cells incubated with FU.

The effect on protein synthesis as measured by TCA precipitable ³⁵S is shown in Fig. 8. In normal cells, as discussed earlier, there is no detectable inhibition of

protein synthesis over about one generation time (shown in Fig. 6). In magnesium starved cells, however, there is a severe inhibition by FU.

In logarithmically growing *E. coli*, FU does not inhibit alkaline phosphatase activity³. In magnesium starved cells (Fig. 9), there is also no marked difference until about 5 h, when the control cells begin to divide. A rather extensive lag in the induction curve of starved cells was also found for the enzyme β -galactosidase.

If the results had been reported as a differential plot, a preferential increase of the enzyme activity in FU-treated cells would have been evident, since both absorbancy increase and general protein synthesis are extensively inhibited. Under limiting conditions, however, preferential enzyme synthesis has been reported^{12,13}. In the present case, because of the low external phosphate concentration, a good deal of the synthetic activity of these cells is dependent upon phosphatase activity. The limiting factor, presumably the supply of "normal" RNA (or precursor) in this case, would be channeled into the synthesis of this enzyme. Ideally, enzyme induction should

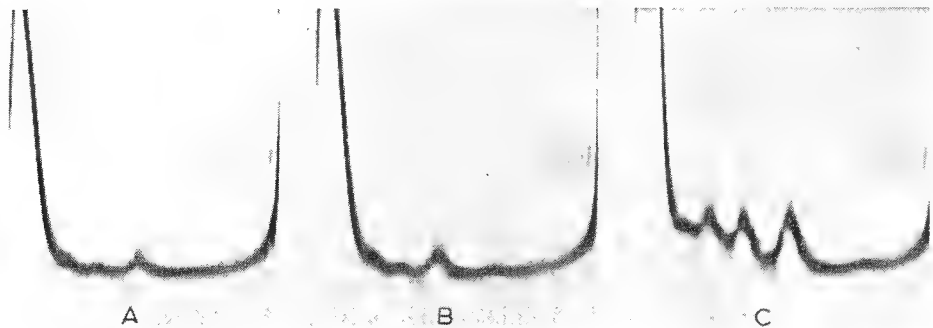


Fig. 7. Schlieren diagrams of crude extracts (40K5S). Pictures taken 5-6 min after reaching speed of 50,740 rev./min. Bar angle, 30°. A, Mg^{++} -starved, 16 h; B, reincubated 150 min with FU; C, reincubated 150 min.

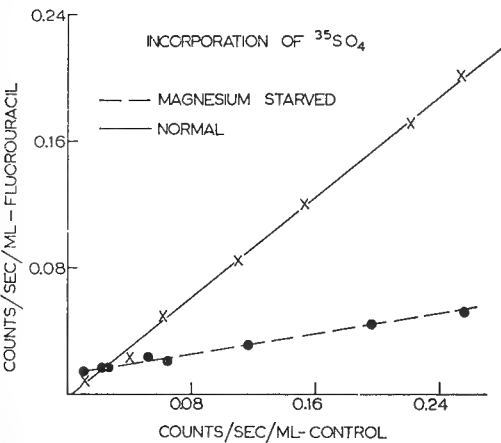


Fig. 8. Comparative rates of incorporation of $^{35}SO_4$ into TCA insoluble fraction of logarithmically growing and magnesium starved cells—in the presence or absence of FU (20 $\mu g/ml$). (Both scales should be increased by a factor of 100.)

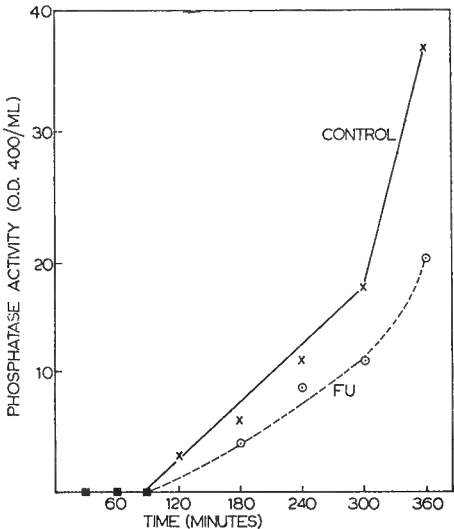


Fig. 9. Increase in alkaline phosphatase activity in magnesium starved bacteria incubated in the presence (20 $\mu g/ml$) and absence of FU.

be measured under conditions of gratuity and would have provided a more valid comparison of the capacity of normal and magnesium starved bacteria.

In addition to depleting the ribosome content, it is of course, conceivable that magnesium starvation is deleterious to other cellular structures that may have to undergo repair before protein synthesis can commence. By inhibiting the necessary reactions, FU would be exerting an indirect but potent effect on protein synthesis.

Starved cells commence protein and nucleic acid synthesis a short time after adding magnesium¹¹. If such an indirect effect were important, therefore, then a short incubation of the cells in the absence of FU should be sufficient to repair any damage and completely eliminate the inhibition on protein synthesis. If, on the other hand, the effect is primarily a direct and immediate one on ribosome synthesis, then addition of the analogue at various times following incubation with magnesium should prevent a further increase in "functional" ribosomes and thus in the rate of protein synthesis.

Following the addition of magnesium there is an exponential increase in ribosomes⁹, and as shown in Fig. 10, there is also an accelerating rate of protein synthesis.

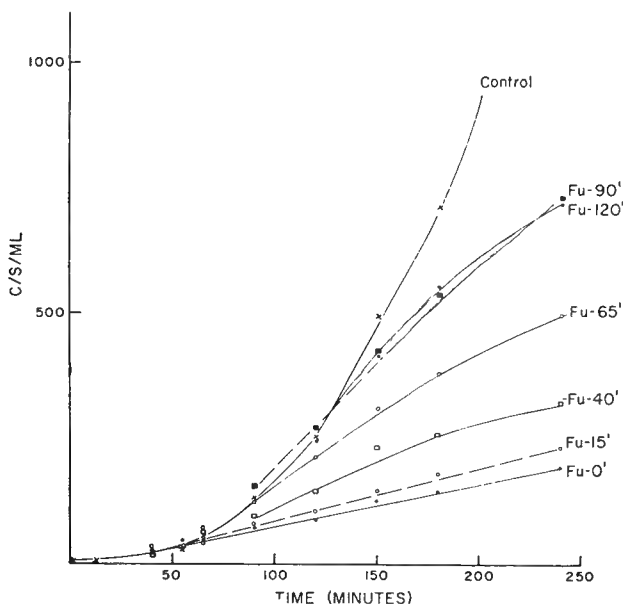


Fig. 10. Effect of FU on $^{35}\text{SO}_4$ incorporation when added at various times following addition of magnesium to starved *E. coli* B.

The addition of FU at various times, however, results in the continued linear synthesis of protein. This experiment suggests that the ribosomes existing at the time of addition of the analogue continue to function but the rate of protein synthesis does not increase because the FU prevents the synthesis of 70-S particles. A more direct action of FU on protein synthesis *via* interruption of "functional" ribosome synthesis would thus be supported.

DISCUSSION

In any analogue studies with intact cells, it is extremely difficult to draw conclusions as to the exact mode of action. This is especially true with fluorouracil where at least

three effects are known: on DNA synthesis¹⁴, on cell wall synthesis¹⁵; and now on RNA metabolism and by inference on protein synthesis.

The effects of FU on RNA and protein synthesis are, however, consistent with other recent observations. On the basis of studies with purine auxotrophs, it is evident that enzymes can be induced with little or no concomitant RNA metabolism¹⁶. In addition, as a result of very short term labelling experiments, McQUILLEN *et al.*⁷ have shown that the larger ribosomes (primarily the 70-S) appear to be the sites of protein synthesis.

Since FU has little or no effect on protein synthesis in actively growing cells, the analogue apparently does not interfere in either the functioning of these larger ribosomes or in the small amount of RNA metabolism that may be simultaneously required.

The primary effect of the analogue appears to be on ribosome metabolism. To interpret the present results, it is assumed that the ribosomes have a major role in protein synthesis. If they do not already exist in the cell, then their synthesis would be mandatory for a given protein to be formed. In two cases studied, phage infected cells and magnesium starved bacteria, it is assumed that the required ribosomes do not already exist and their synthesis should, therefore, be a necessary precursor of protein synthesis. In both cases, FU inhibited protein synthesis, suggesting that the possible correlation between inhibition of ribosome metabolism and of protein synthesis is meaningful.

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IV.B.6 5-Fluorouracil

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J. E. M. Midgley

5-Fluorouracil

It has been found by Chargaff et al. that, in the presence of small amounts of 5-fluorouracil (FU) in the absence of added uracil, the *E. coli* mutant 15^{TAU-}, which requires thymine (T), arginine (A), and uracil (U), will continue to incorporate S³⁵ into the protein of the cell for periods up to 1 hour. Little of the FU was found to enter the cell during exposure to the analog (Year Book 59).

These experiments were continued to determine where this small amount of analog exerted its effect in the cell. Depletion of the exogenous uracil was achieved by growing the mutant with a small quantity of uracil, sufficient to allow growth of the cells for about 20 minutes, after which growth and the rate of protein synthesis declined markedly. After 10 minutes of uracil depletion, 5-fluorouracil (20 mg/l) was added to the culture. The absorption of the culture at 650 m μ increased shortly afterward, indicating further growth.

C¹⁴-labeled FU was used to measure incorporation into the ribosomal RNA of the cells. After 10 minutes' incorporation, the cells were broken and analyzed on DEAE. Figure 60 shows that the analog entered the cell but that it remained unassociated with the ribosomal RNA. Almost the whole of the incorporated analog eluted at 0.25 M, only about 2 per cent being incorporated into the ribosomes. It was also of interest to note that only one type of compound containing the analog was produced in any quantity.

A similar experiment was performed using P³²O₄⁼. In this experiment the label was added 10 minutes after addition of the analog to the medium. Figure 61 indicates

the presence of two peaks, not prominent in normal growing cells. One of these (Z) is probably identical to the peak observed with C¹⁴-fluorouracil, eluting at 0.25 M NaCl. The other (Y) elutes at 0.185 M NaCl, and has 1.5 times the P³² in it as the Z peak.

To determine whether these compounds were originally attached to ribosomal material, P³²-labeled cell juices were centrifuged (40K 180) to remove most of the ribosomal RNA. Analysis on DEAE showed that the supernatant fluid contained as much of the two substances as the whole cell extract. In contrast, the ribosomal pellet contained very little (less than 1 per cent) of the peaks Y and Z.

The nature of these materials was next investigated. The use of Sephadex G25 indicated that the molecular weight of both Y and Z is under 2000, and their retention times were almost identical, indicating similarities in size. Two-dimensional chromatography of the cell juice showed several compounds present after 10 minutes' labeling with P³²O₄⁼ in +T, +A, +FU conditions as diagrammatically represented in figure 62. A pulse and chase were also carried out to determine the turnover of the materials detected by autoradiography. The first spot to indicate radioactivity was I, probably ATP. Within 3 minutes, spots Y, Z, and II also appeared in the autoradiogram. The positions of Y and Z were checked by comparing an autoradiogram of purified Y and Z. Upon chasing with P³¹O₄⁼, spots I and II quickly disappeared, but Y and Z were stable and remained throughout the period of chasing. It thus appears that the phosphorus in the Y and Z peaks does not turn over rapidly once the compounds are formed, in

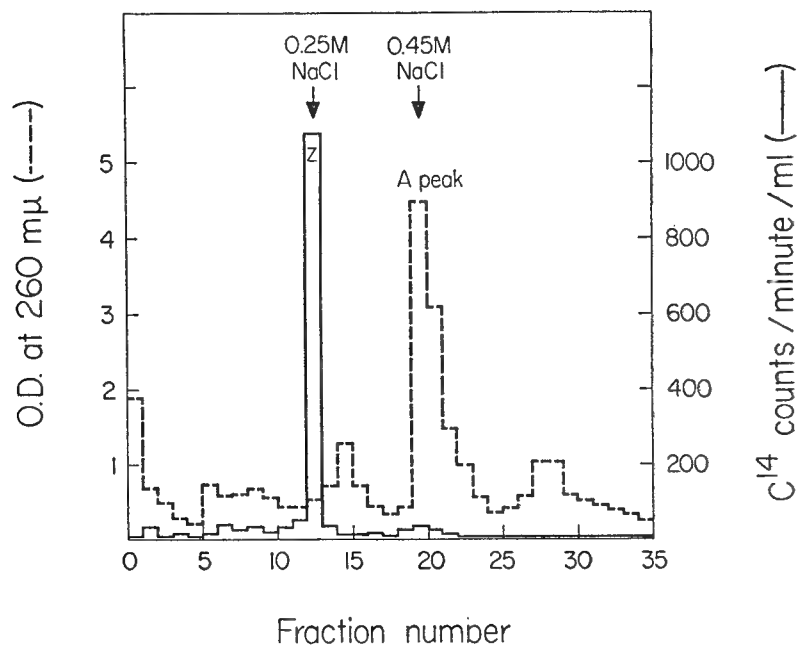


Fig. 60. DEAE analysis of a complete broken-cell extract of 15^{TAU-} after 20 minutes' growth in the presence of C^{14} -fluorouracil (20 mg/l). Note radioactivity in peak (Z) eluting at 0.25 M and lack of radioactivity in ribosome (A) and ribosome precursors.

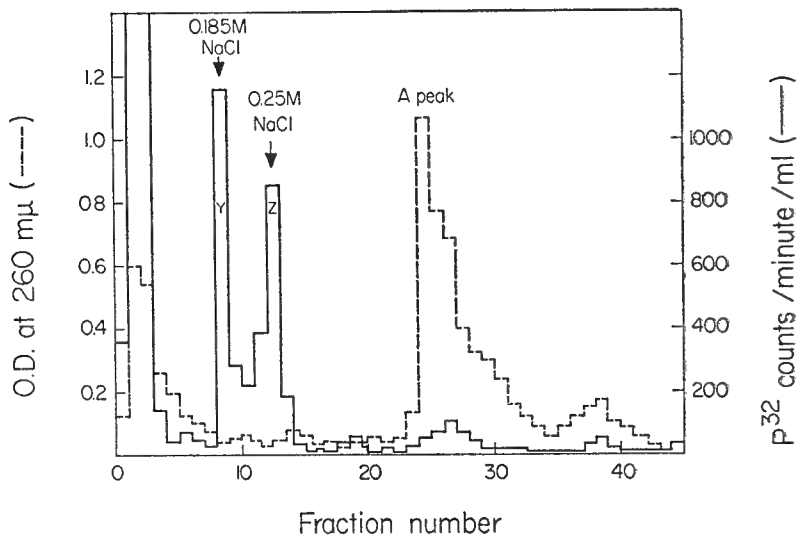


Fig. 61. DEAE analysis of complete cell juice from 15^{TAU-} grown in +T, +A, +5-FU, -U conditions for 10 minutes in the presence of P^{32} .

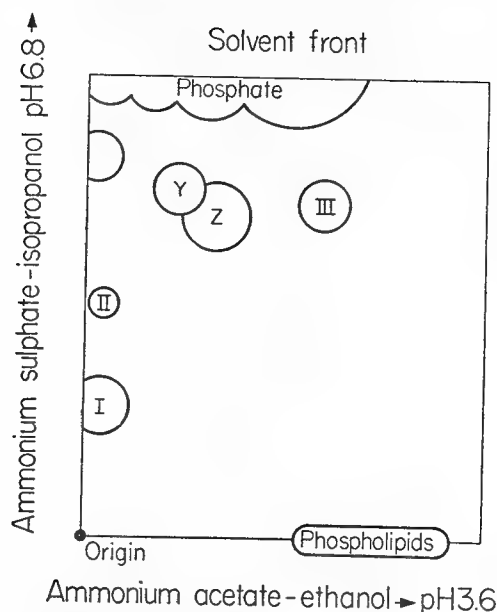


Fig. 62. Two-dimensional chromatogram of substances labeled by P^{32} in a 40K 240 supernatant of broken 15^{TAU-} cells, during a 10-minute period of growth in +T, +A, +5-FU, -U medium. Twenty milligrams per liter of 5-FU was supplied after growth in a +T, +A, +U medium had slowed as the result of the running out of U. Five minutes later, P^{32} was added and samples were taken during a 30-minute period.

contrast to the labile phosphorus molecules of ATP, etc.

From the position of the spots Y and Z on the chromatogram, it was suspected that both materials might contain pyrimidine nucleotides. HCl hydrolysis followed by chromatography in ammonium sulfate indicated that Y contained uracil, and that Z contained FU. The high ratio of P^{32} /UV, the position of the materials in two-dimensional paper chromatography, and the ready liberation of some of the P^{32} on gentle hydrolysis suggest that the compounds may be 5-polyphosphate forms.

In the presence of 5-fluorouracil, many strains of bacteria accumulate cell-wall derivatives such as UDP-N-acetylglucosamine amino acids. Accordingly, $S^{35}O_4^{2-}$ was substituted for $P^{32}O_4^{3-}$ to detect the presence of sulfur amino acids. DEAE analysis of broken-cell extracts (40K 180

SN) indicated that both Y and Z contained an S^{35} component in addition to the nucleotide components (fig. 63). A similar C^{14} amino acid mixture gave identical radioactive peaks. In both cases, however, it was necessary to precipitate protein which otherwise would obscure the Y and Z peaks. Possible turnover of S^{35} in the Y and Z peaks was next investigated by the use of a 10-minute pulse of $S^{35}O_4^{2-}$ in +T, +A, +FU, -U conditions followed by a chase using excess S^{32} methionine and S^{32} cysteine. The chase was carried out for 10 minutes. During incorporation of S^{35} , radioactivity appeared in glutathione, Y and Z. S^{35} also entered the proteins of the cell. Upon chasing, the radioactivity in the Z peak disappeared within 40 seconds, but that of the Y peak and glutathione remained almost unchanged. After 10 minutes, the S^{35} in the Y peak had also been chased out, leaving radioactivity still in the glutathione. There is thus some evidence that amino acids of Y and Z peaks turn over, the Z component turning over faster than the Y. As the amounts of Y and Z are unknown, and their rate of turnover is as yet only crudely determined, nothing can be said to relate the turnover of S^{35} in them with the entry of S^{35} into protein.

A comparison was next made between the DEAE elution pattern of 15^{TAU-} in +T, +A, +FU, -U conditions and that of *E. coli* strain B in media containing the same quantity of analog. Figure 64 shows the DEAE analysis of P^{32} -labeled material from broken whole cells of *E. coli* B given 10 minutes' exposure in the presence of 5-fluorouracil (20 mg/l). Although a new peak appears which elutes at 0.145 M NaCl, no peaks are found at 0.18 M or 0.25 M. Also, the amount of P^{32} entering the ribosomal peak is much greater than with 15^{TAU-} . One of the most noteworthy features of 15^{TAU-} grown in +FU, -U conditions is the relative absence of P^{32} in the ribosomes, or indeed in any ribosomal precursors.

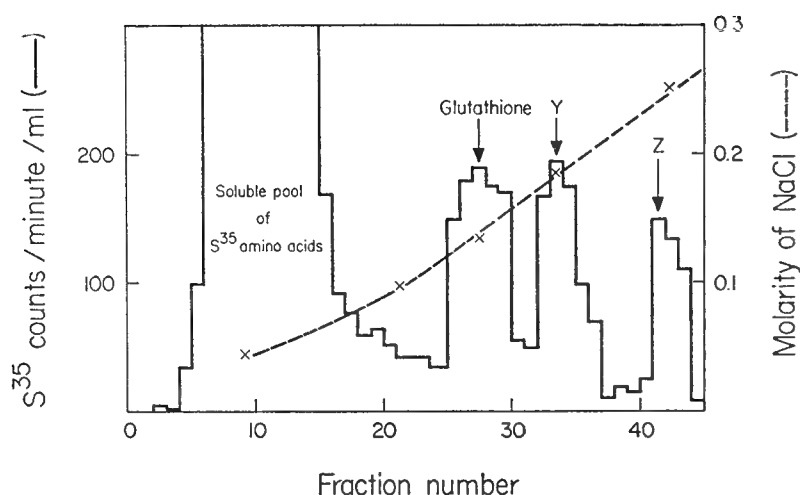


Fig. 63. DEAE analysis of TCA-soluble components of cell juice. The cells (15^{TAU-}) were grown in +T, +A, +5-FU, -U conditions in the presence of $S^{35}O_4=$ for 20 minutes. Label also entered the amino acids of protein, which was precipitated by 5 per cent TCA to prevent obscuration of peaks Y and Z.

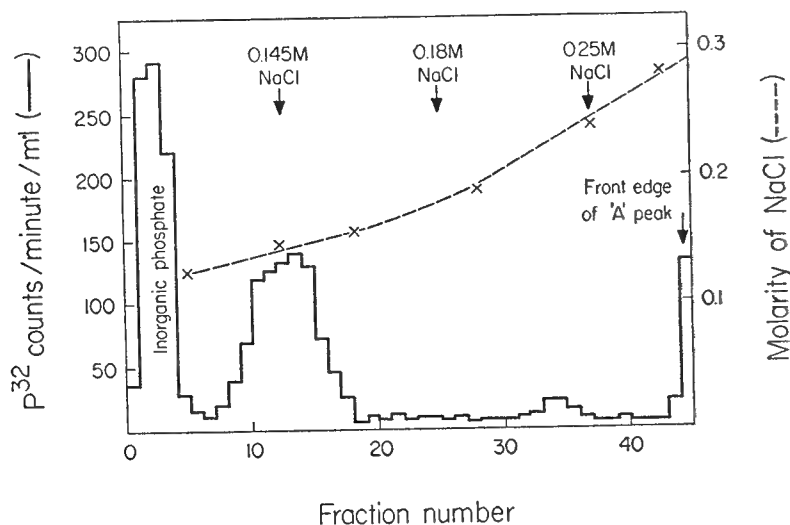


Fig. 64. DEAE analysis of material from *E. coli* B cells grown in the presence of 5-FU and P^{32} . No peaks eluting at 0.18 M and 0.25 M NaCl are visible.

In view of the widespread effects of 5-fluorouracil on the metabolism of micro-organisms, it is difficult to decide where the analog is acting to allow protein synthesis to continue. Similar amounts of the natural uracil allow synthesis for only a

short period. Since proteins are formed without concurrent ribosome synthesis when Y and Z have accumulated, these compounds seem to deserve further study to determine whether they play any role in protein synthesis.

Comment. This study was not brought to completion, partly because of the difficulties in working with compounds present in such small quantities and partly because Midgley became engrossed in resolving the controversy over the composition of newly formed RNA. The prolonged protein synthesis caused by the addition of 5 Fu remains an unsolved problem. It implies that templates can function for long periods of time and that protein synthesis may require a cofactor not recognized as yet. Richard B. Roberts.

C. Other Aspects of Protein Synthesis

IV.C.1 The Effects of a Tryptophan-Histidine Deficiency in a Mutant of Escherichia coli

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Cells placed in a favorable environment are capable of duplicating themselves indefinitely. This implies that the over-all reaction rates of each of the synthetic systems must be balanced as every component of the cell is duplicated in the same time interval. It is evident that the various systems must be somehow coupled together since completely independent systems could not maintain these balanced rates in various environmental conditions. Thus, a coupling between the carbohydrate metabolism and synthetic activities is demonstrated by the decrease in oxygen uptake when nitrogen is removed from the medium. At the same time there is a degree of independence among the various metabolic systems. For example, carbohydrate metabolism proceeds at a reduced rate even though growth is stopped by lack of nitrogen. Also, certain ion deficiencies have more pronounced effects on some synthetic activities than on others.

We have attempted to examine the degree of coupling among the various systems by observing the metabolism of mutant cells of *Escherichia coli* which were prevented from duplicating by the lack of one or more required amino acids. This deficiency would be expected to block protein synthesis, but the immediate effect on other systems, in particular nucleic acid synthesis, could not be predicted. Radioactive carbon, sulfur, and phosphorus were utilized as indicators of synthesis; growth and respiration were observed by the usual techniques. On completing these experiments it became apparent that the method utilized in this work might also be of value in determining the mode of action of various poisons and antibiotics.

METHODS

A mutant of *E. coli*, strain B, requiring tryptophan and histidine (TH) was obtained by using the Davis technique (Davis, 1949) on the mutant B/r/1, tryptophan (Anderson, 1946). The same technique was used to isolate a lysine requiring mutant from B/r (Witkin, 1947). To avoid difficulties from reversions to wild type most of the experiments were conducted with the tryptophan-histidine mutant. Cells were grown overnight on a synthetic medium, M-9, (Anderson, 1946) containing sodium chloride, sodium-potassium phosphate buffer, ammonium chloride, magnesium sulfate, and sufficient glucose (1.2 g per liter) to give 1 g wet weight of cells per liter. The required amino acids were added to this basic medium in a concentration sufficient for maximum growth. Under these conditions, growth stops abruptly when the glucose supply is exhausted and resumes without a lag when the cells are washed and placed in a fresh complete medium. Before use in experiments, cells were harvested by centrifuging, then washed twice with 0.85 per cent sodium chloride.

The radioactive isotopes were obtained from the Atomic Energy Commission at Oak Ridge. The radioactive phosphorus, received as $H_3P^{32}O_4$, was hydrolyzed in HCl before use to convert any other phosphate ions which might be present as contaminants to the orthophosphate form. S^{35} was used as sulfate. C^{14} was received as barium carbonate and converted to $NaHC^{14}O_3$. Approximately 1 millicurie of tracer was used per liter of medium. This quantity is adequate for counting and should have no harmful effect on the cells since the total radiation received is less than 5 roentgens (1/1,000 of the mean lethal dose). Radioactive samples were measured with a Geiger counter correcting, if necessary, for self-absorption. With P^{32} and S^{35} present simultaneously, each sample was measured with and without a cover of aluminum foil. The individual contributions of the two radioelements could be calculated since the foil absorbed 90 per cent of the radiation from the S^{35} and 5 per cent of the radiation from the P^{32} .

Cell concentrations were determined by measuring the optical densities of suspensions with a Beckman spectrophotometer at 650 $m\mu$.

TABLE 1
Growth of tryptophan-histidine mutant of Escherichia coli

HISTIDINE (MG/ML)	TRYPTOPHAN (MG/ML)				
	0	0.002	0.004	0.010	0.020
0	0	0	0	0	0
0.002	0	0.18*	0.22	0.25	0.20
0.004	0	0.21	0.28	0.34	0.47
0.010	0	0.21	0.31	0.57	0.53
0.020	0	0.19	0.30	0.60	0.60

* Cells (millions) per liter medium after 17 hours' incubation.

RESULTS

*Growth.*¹ Test tubes containing 2 ml of M-9 and varying concentrations of tryptophan and histidine were lightly inoculated with a washed suspension of mutant cells. After 17 hours' incubation at 37 C without aeration the optical densities were measured. Table 1 shows that growth is limited by a deficiency of either tryptophan or histidine.

When large inocula of washed cells (tryptophan-histidine) were placed in a medium lacking the required amino acids, there was a period of slow growth. During the first two hours the optical density increased at a rate of 6 per cent per hour. After four hours the rate dropped to 3 per cent per hour and after 6 hours there was no further increase. Plate counts showed that the number of viable cells increased by a factor of two during the first four hours. The observed increase in the mass and number of cells was due to the tryptophan-histidine mutants, as samples plated on M-9 showed that less than 0.1 per cent of the population was capable of forming colonies on media lacking histidine and tryptophan.

¹ "Growth" is used to indicate any increase in cell mass of the culture. "Duplicate" is used for the special type of growth where the cells duplicate themselves indefinitely.

Respiration: The oxygen uptake of the tryptophan-histidine mutant was observed using a Warburg respirometer. Table 2 shows that the respiration rate was increased by the addition of ammonium chloride even though the required amino acids were not present. When both ammonium chloride and the amino acids were added, there was a further increase in the respiration rate.

Sulfur: Synthesis of protein was measured by the incorporation of radioactive sulfur. Washed cells were distributed to tubes containing M-9 and radiosulfate

TABLE 2
Relative rates of respiration of tryptophan-histidine mutant of Escherichia coli in various media

SUPPLEMENTS TO NITROGEN-FREE MEDIUM			
No supplement	Tryptophan and histidine, 0.02 mg/ml	Ammonium chloride, 1.4 mg/ml	Tryptophan and histidine + ammonium chloride
1.0	1.3	1.4	2.6

TABLE 3
Radiosulfur uptake (counts/second) by tryptophan-histidine mutant of Escherichia coli

	TIME (HR)	SUPPLEMENTS TO BASAL MEDIUM—0.020 MG EACH PER ML						
		(1) —		(2) Histidine		(3) Tryptophan		(4) Histidine and tryptophan (c/s)
		c/s	% of (4)	c/s	% of (4)	c/s	% of (4)	
Cold trichloroacetic acid soluble	0	2		1		3		1
	1	18	(16)	23	(21)	16	(15)	110
	2	35	(15)	31	(13)	33	(14)	236
Trichloroacetic acid insol- uble (protein)	0	2		1		3		2
	1	21	(5.8)	22	(6)	26	(7)	364
	2	32	(5.5)	48	(8.2)	36	(6.2)	582
Cells (millions) per liter								
Concentration of cells	0	1.4		1.4		1.4		1.4
	1	1.6		1.6		1.5		2.1
	2	1.7		1.7		1.6		2.5

and aerated at 37 C. At intervals, samples were removed and the optical density was measured. These samples were washed with 0.85 per cent NaCl, centrifuged, and the dry pellets of cells frozen. At the end of each experiment, the frozen pellets were extracted for one-half hour with cold 5 per cent trichloroacetic acid and centrifuged. The trichloroacetic acid precipitate contained the sulfur incorporated into proteins (Bolton, Cowie, and Sands, 1952). Table 3 shows that in the absence of either required amino acid, protein synthesis is reduced to less than one tenth of the normal rate.

Phosphorus: Radioactive phosphorus was used to observe the synthesis of

nucleic acids. The initial procedure was the same as with radiosulfur; in addition the trichloroacetic acid precipitate was further fractionated by a modification of

TABLE 4
Radiophosphorus uptake (counts/second) by tryptophan-histidine mutant of Escherichia coli

	TIME (HR)	SUPPLEMENTS TO BASAL MEDIUM—0.020 MG EACH PER ML						
		(1) —		(2) Histidine		(3) Tryptophan		(4) Histidine and tryptophan (c/s)
		c/s	% of (4)	c/s	% of (4)	c/s	% of (4)	
Desoxypentose nucleic acid	0	0		0		0		1
	1	7	(23)	10	(33)	7	(23)	30
	2	16	(28)	22	(38)	16	(28)	53
Pentose nucleic acid	0	1		0		0		0
	1	14	(13)	18	(17)	16	(15)	106
	2	40	(22)	42	(24)	43	(24)	177
Alcohol soluble (lipids)	0	0		0		0		1
	1	17	(50)	21	(61)	11	(32)	34
	2	23	(31)	32	(44)	31	(42)	73
Cells (millions) per liter								
Concentration of cells	0	0.93		0.97		1.0		0.99
	1	1.0		1.0		1.1		1.2
	2	1.1		1.2		1.1		1.4

TABLE 5
Radiocarbon uptake (counts/second) by tryptophan-histidine mutant of Escherichia coli

	SUPPLEMENTS TO BASAL MEDIUM—0.020 MG EACH PER ML						
	(1) —		(2) Histidine		(3) Tryptophan		(4) Histidine and tryp- tophan (c/s)
	c/s	% of (4)	c/s	% of (4)	c/s	% of (4)	
Hot trichloroacetic acid soluble (nu- cleic acid)	65	(18)	99	(27)	84	(23)	358
Trichloroacetic acid insoluble (pro- tein)	59	(11)	76	(14)	59	(11)	536
Alcohol soluble	40	(200)	61	(305)	45	(225)	20
Cells (millions) per liter							
Concentration of cells	0	0.89	0.89	0.91	0.54		
	1 hr	0.89	0.93	0.92	0.97		

the Schmidt-Thannhauser method (1945). After one hour extraction with 95 per cent ethanol at 20 C to remove the lipids, the precipitate was digested for 18 hours with 1 N KOH. Finally, precipitation with trichloroacetic acid separated the desoxypentose nucleic acid from the pentose nucleic acid.

Table 4 shows that when either required amino acid is omitted from the me-

dium, the incorporation of phosphorus into nucleic acids is reduced to roughly one fourth.

Sulfur-phosphorus: Experiments with sulfur and phosphorus in parallel cultures consistently showed that the omission of one of the required amino acids reduced protein synthesis by a factor of ten and synthesis of nucleic acid by a factor of four. To ensure identity of conditions, radiosulfur and radiophosphorus were both added to the same tubes. The procedure followed was the same as in the radiophosphorus experiments. Although these radioactivity measurements are less accurate, the results were the same as those of tables 3 and 4.

Carbon: The syntheses of nucleic acids and protein were also studied by measuring the incorporation of radiocarbon. Washed cells were shaken for one hour at 37 C in the presence of $C^{14}O_2$. Following this, the cells were washed and the lipids and carbohydrates removed by extraction with hot 80 per cent ethanol and an ethanol-ether mixture. The ethanol precipitate was then treated with 5 per cent trichloroacetic acid for twenty minutes at 100 C; the soluble fraction contained nucleic acid while the trichloroacetic acid precipitate contained the proteins. This completely independent method gives results (table 5) which confirm those obtained with radiosulfur and radiophosphorus in showing that the amino acid deficiency causes a greater reduction of protein synthesis than of nucleic acid synthesis.

Alternation of amino acids: In one experiment the cells were incubated for one hour with tryptophan and then washed and incubated for the second hour with histidine. The phosphorus uptake was the same as during two hours' incubation with tryptophan alone. Evidently, there is very little storage of free amino acids or peptides, and both amino acids are required simultaneously (Swanson and Clark, 1950).

Exchange: Mutants grown in the presence of radiophosphorus and radiosulfur and subsequently harvested, washed, and incubated for two hours in M-9 showed a loss of radioactivity. The trichloroacetic acid soluble P^{32} decreased by 50 per cent while the nucleic acid P^{32} decreased by 5 per cent. The protein bound S^{35} showed a similar decrease of 5 per cent. The loss of P^{32} from the trichloroacetic acid soluble fraction is smaller than the loss observed in normally grown cells. This could be expected from the observed lower rate of oxygen uptake. However, the 5 per cent loss of nucleic acid P^{32} and protein S^{35} is greater than the loss observed with normally growing cells which show no loss from these fractions (Roberts and Roberts, 1950; Cowie, Bolton, and Sands, 1950). Consequently, this loss probably is due to the degradation of cellular components.

Lysine mutant: The lysine deficient mutant gave similar results in experiments on growth, respiration, and the incorporation of sulfur and phosphorus. In addition, the rate of adaptation to a new energy source, arabinose, was observed. This adaptation process required the same time whether or not lysine or ammonium chloride was present.

DISCUSSION

The mutants used in these experiments do not duplicate when they lack a required amino acid. However, in a dense culture, some of their metabolic activities

proceed at reduced rates even in the absence of the required amino acids. For several hours, the cell mass increases; the number of cells also increases indicating cell division. Synthesis of cellular components continues as shown by the incorporation of radioactive indicators. Respiration also continues although at a reduced rate. These processes are not due to traces of the required amino acids remaining from the original protein media, as the rates remain constant during the first two hours. Neither can they be ascribed to bacterial contaminants as this possibility is ruled out by plate counts. Growth eventually stops after six hours probably because the balance among the various components of the cell is too severely disturbed. In the absence of the required amino acids, 10 per cent or less of the normal protein synthesis takes place. This small residual synthetic activity is probably due to the utilization of tryptophan and histidine released from degraded proteins. The S^{35} released by the cells indicates that 5 per cent of the proteins are degraded; this quantity should be sufficient to supply the tryptophan and histidine required for the observed synthesis. The rates of synthesis and degradation appear to be equal with the total protein remaining constant.

The C^{14} radioactivity observed in the alcohol soluble fraction was actually higher when the amino acids were omitted. Free amino acids were not found in the medium, and Taylor (1947) has shown that they are not accumulated in *E. coli*. Consequently, it seems that when the amino acids are not utilized for protein synthesis they are no longer formed, and their precursors accumulate.

When the required amino acids are missing, nucleic acid synthesis as measured by phosphorus or carbon is reduced by a factor of four. As tryptophan and histidine are not recognized as precursors of nucleic acid, it would appear that this reduction in the nucleic acid synthesis is a secondary effect of the primary blockage in protein synthesis. Protein synthesis and nucleic acid synthesis are, however, somewhat independent since the conditions which reduce the rate of protein synthesis by a factor of ten reduce nucleic acid synthesis by a factor of four.

The residual growth and the oxygen uptake observed when the required amino acids are missing indicate again that some of the synthetic activities of the cells continue at reduced rates even when the protein synthesis is blocked.

SUMMARY

The metabolism of a mutant of *Escherichia coli* requiring tryptophan and histidine was compared in media with and without these amino acids. Synthesis of cellular components was observed using C^{14} , S^{35} and P^{32} as indicators. In the absence of the amino acids, protein synthesis is reduced to 10 per cent of normal while nucleic acid synthesis is less affected being 25 per cent of normal. Protein synthesis and nucleic acid synthesis are thus partially independent.

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Comment. The time scale used in these experiments shows how little comprehension we had in 1952 of the time required for protein synthesis and the magnitude of the supply of intermediates. The experiments did yield an early observation of the requirement for amino acids in nucleic acid synthesis.

Richard B. Roberts.

IV.C.2 Role of Peptides in Protein Synthesis

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R. B. Roberts

ROLE OF PEPTIDES IN PROTEIN SYNTHESIS

One theory of protein synthesis holds that amino acids are bound together into small peptides, and that the small peptides are linked into larger peptides and finally linked into the polypeptide chain of the protein. According to these ideas one might expect to find peptides as intermediates of protein synthesis; cells in which protein synthesis was blocked might accumulate peptides; and the cells might prefer to incorporate peptides rather than free amino acids. Several experiments were carried out during the year to test these possibilities.

A group of C^{12} glycine peptides were used as isotopic competitors with C^{14} glucose. Chromatograms showed that the peptides supplied all the glycine required by the cell as well as all the other amino acids of the peptide. Thus, if C^{12} glycyl-

leucine was present, neither glycine nor leucine contained C^{14} from glucose. As only a small portion of the protein-bound glycine or leucine is in the form glycyl-leucine, it is evident that the peptide was rapidly broken down to the free amino acids and used largely after degradation. Chromatography of the medium also showed that the peptide was almost completely split. Similar results were obtained with cysteinylglycine, glycylalanine, glycylphenylalanine, glycylglycine, and triglycine. Specific incorporation of labeled peptides might be possible to observe, but the isotopic competition experiments do not encourage anyone to undertake the major effort required to isolate the peptides. It is obvious that the incorporation would be very inefficient and that most of the peptides would be degraded to free amino acids.

TABLE 17
INCORPORATION OF HYDROLYZATES OF C¹⁴-LABELED PROTEIN

TRACER	COMPETITOR	RADIOACTIVITY INCORPORATED	
		Nucleic acid	Protein
C ¹⁴ protein partial hydrolyzate.....	None	78	520
	C ¹² amino acid mixture	45	370
C ¹⁴ protein complete hydrolyzate.....	None	148	1320
	C ¹² amino acid mixture	56	520

One attempt was made to observe small peptides as a cellular component. A careful examination of the TCA-soluble fraction of the cell showed that unusual movements on paper chromatograms of a number of ninhydrin-positive spots, previously suspected to be peptides, were actually due to the presence of traces of TCA. When the TCA was completely removed by using a Dowex 50 ion-exchange column, all the observed ninhydrin-positive material could be identified as free amino acids and glutathione.

Neither could any accumulation of peptides be observed in metabolically blocked cells which could not synthesize protein. Nitrogen-, sulfur-, and magnesium-deficient media were used to prevent protein synthesis. In no case could any peptide accumulation be observed. These media were also used to look for exchange of free amino acids with protein-bound amino acids. No exchange was found.

Two mutants were also used, one requiring phenylalanine and the other methionine. These mutants could not synthesize protein in the absence of the one required amino acid, but peptide did not accumulate, either in the cell or in the culture fluid.

Having obtained only negative evidence on peptide utilization, we thought it de-

sirable to repeat again the one kind of experiment which has shown an indication of preferential utilization of peptides. A culture of *E. coli* cells was grown in the presence of C¹⁴ glucose to give uniformly labeled protein. One half of this protein was hydrolyzed briefly, and a chromatogram showed that the hydrolyzate contained mostly large peptides with only a small quantity of free amino acids. The other half was hydrolyzed 18 hours to free amino acids. These hydrolyzates were then used to supplement glucose media both with and without a large excess of unlabeled free amino acids. The results (table 17) agree completely with unpublished experiments of Abelson carried out in 1952. The addition of C¹² amino acids did not reduce the incorporation of radioactivity from the partial hydrolyzates as much as it did that from the complete hydrolyzate. Evidently there is some preferred incorporation of amino acids found in large peptides. In view of the other negative data on peptide accumulation and the observed adsorption of free amino acids, this preferential utilization of large peptides does not seem to be of major importance in protein synthesis. At present it appears that the template idea is more nearly correct than the peptide idea.

Comment. Year Book 60 also carries kinetic evidence that peptides are (not intermediates in protein synthesis (see II.B.1). The preferential uptake observed is probably due to preferential entry into the cell (E. Ball, J. Humphreys, and E. Shive, Arch. Biochem. Biophys., 73, 410-419, 1958). Richard B. Roberts.

IV.C.3 The Induced Synthesis of β -Galactosidase in *E. coli*, 1, Synthesis of Enzyme under Various Experimental Conditions*

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INTRODUCTION

The mechanism involved in induced synthesis of enzymes (enzymic adaptation) in microorganisms has been paid very great attention because of its importance for the understanding of the ways in which the living protoplasm directs and controls the synthesis of proteins (enzymes).

The induced synthesis of β -galactosidase in *E. coli* is probably the most thoroughly investigated case of enzyme adaptation. The reason for this preference is particularly the introduction, by LEDERBERG¹, of a very simple photometric method for determination of the activity of this enzyme, using *o*-nitrophenyl- β -D-galactoside as substrate.

One of the most fascinating features of induced enzyme synthesis is that it may occur even in the absence of any exogenous nitrogen source. Under these conditions growth is completely arrested, and it might be expected that any kind of protein synthesis was also inhibited. However, a certain amount of low-molecular nitrogen reserves, consisting mainly of amino acids, has been demonstrated in yeast (ROINE²), and this reserve is being utilized when adaptation occurs in the absence of nitrogen (HALVORSON AND SPIEGELMAN³). In the present work it has been attempted to demonstrate some of the factors influencing the rate and extent of the induced synthesis of β -galactosidase, especially in the absence of exogenous nitrogen.

MATERIAL AND METHODS

Biological material. All experiments were done on *Escherichia coli*, strain B. For details about culture methods, media, etc. the reader may refer to ROBERTS *et al.*⁴.

Extraction of enzyme. Samples taken from a bacterial suspension (25-100 ml, depending on the enzyme content) were centrifuged and the supernatant discarded. The enzyme was liberated by shaking the pellet of bacteria in 10 ml distilled water + 0.25 ml toluene for one hour at 37° C (COHN AND MONOD⁵). The resulting extracts were not centrifuged, as about 50% of the enzyme activity was found to be bound to the precipitate. These extracts were then appropriately diluted.

* The work presented in this and the following paper was done during the tenure of a Special Fellowship of the Carnegie Institution of Washington 1951-52.

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Preparation of substrate. LEDERBERG's substrate¹, *o*-nitrophenyl- β -D-galactoside (ONPG) was synthesized in the following steps: D-galactose was transformed to pentaacetyl- β -D-galactose⁶, from which was made tetraacetyl- α -D-galactopyranosyl bromide (HAYNES AND TODD⁷). This substance was transformed *via o*-nitrophenyl- β -D-galactopyranoside tetraacetate, into ONPG (SEIDMAN AND LINK⁸).

Determination of enzyme activity. The procedure adopted was essentially a micromodification of the method described by LEDERBERG¹. To a small test tube was added 200 μ l of a buffered salt solution (*cf.* below), 25 μ l enzyme solution, and 30 μ l 0.005 *M* ONPG*. After 20 minutes incubation at 37° C, the reaction was stopped and the yellow colour of the liberated *o*-nitrophenol developed by adding 25 μ l 1 *M* K₂CO₃. The absorbancy was read at 420 *m* μ in the Beckman spectrophotometer, using Pyrocell microcuvettes.

At the time the present experiments were performed, the paper by LEDERBERG¹ was the only one available, dealing with the activation of β -galactosidase. LEDERBERG found that mono-valent cations were necessary to activate this enzyme, and that Na⁺ was the best activator. Experiments designed to demonstrate the effect of various salt solutions on the activity of the enzyme showed that approximately the same activity was found in *M*/25 Na-phosphate-buffer, pH 7.5; 0.1 *M* NaCl, and the salt solution in which the bacteria were grown ($8.5 \cdot 10^{-2}$ *M* Na⁺; $2.2 \cdot 10^{-2}$ *M* K⁺, pH 6.8). The latter solution was therefore used in all experiments. The results are expressed by the amount of ONPG split, as μ M \times minute⁻¹ per mg dry weight (DW). The dry weight was calculated from readings of the optical density of the bacterial suspension, employing an empirical curve correlating density and cell volume (wet weight), and assuming that the dry weight is 25 % of the wet weight.

RESULTS

Enzyme synthesis in complete and in nitrogen-free media

The course of enzyme synthesis in a complete medium, in which the energy source

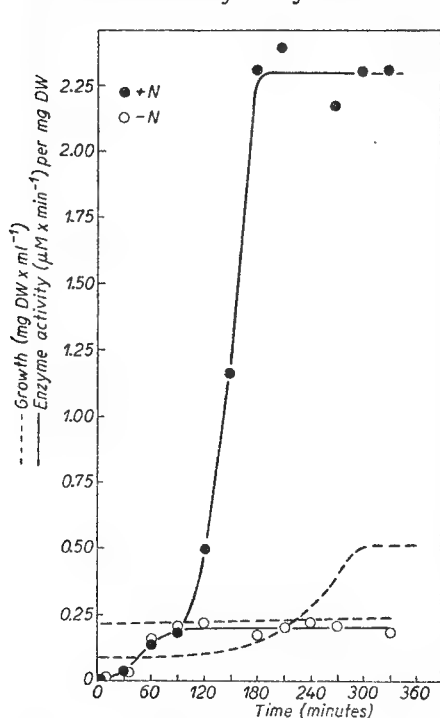


Fig. 1. Growth and induced enzyme synthesis in the presence and absence of nitrogen.

is lactose (1 mg \times ml⁻¹), may be seen in Fig. 1. In this figure both concentration of enzyme and the growth have been plotted against time. It is seen that there is a lag phase in the growth lasting about 90 minutes. Contrary to this, enzyme synthesis begins immediately, and at the end of the lag phase the concentration is about 0.20 μ M \times min⁻¹ per mg DW. It should be noticed that, confirming LEDERBERG's results, a slight amount of β -galactosidase was found even in the unadapted cells, varying between 0.003 to 0.005 μ M \times min⁻¹ per mg DW. When the cells start growing, at the end of the lag phase, a very rapid enzyme synthesis simultaneously begins. The enzyme synthesis is not proportional to the increase in cell number (DW). Calculating the ratio: Increase in total enzyme/increase in DW, for time intervals shortly after the lag phase, 90–120, 120–150 minutes *etc.*, have for several curves given values in the range 4–7 μ M \times min⁻¹ per mg DW. Plotting these two quantities in a graph shows that after 1–2 hours of growth linearity is approached between them, the slope corresponding to

* My heartiest thanks are due to Professor J. LEDERBERG for a sample of ONPG.

$1.75\text{--}2.25 \mu\text{M} \times \text{min}^{-1}$ per mg DW for several experiments. This linearity is also indicated in Fig. 1. by the constancy of enzyme activity per mg DW found after 3 hours. The final concentration of enzyme in the cultures after adaptation to lactose ranged in most experiments also between 1.75 and $2.25 \mu\text{M} \times \text{min}^{-1}$ per mg DW, although occasionally even higher and lower values were observed. The DW employed in the latter values includes also the bacteria added at the beginning of the experiment, which are not negligible compared to the final amount (*cf.* the growth curve in Fig. 1).

The linear relationship between growth and enzyme synthesis mentioned above may represent the normal content of enzyme in already adapted bacteria. If this be true, the amount of enzyme at the end of the experiment may be accounted for by assuming that both old and new cells synthesize the enzyme.

Comparing enzyme synthesis in the complete medium and in the N-free medium, it is seen that these processes proceed similarly during the first 90 minutes, *i.e.* during the lag phase (Fig. 1). In both cases the rate of synthesis is gradually increasing during the first 30 minutes. After 90 minutes no further synthesis occurs in the N-free medium, the enzyme content remaining constant at a level about $0.2 \mu\text{M} \times \text{min}^{-1}$ per mg DW during the rest of the experiment. As enzyme synthesis during the lag phase thus is the same in both media, it seems reasonable to suggest that the endogenous nitrogen reserves during this time are the only ones available for protein synthesis even in the complete medium.

Enzyme synthesis during diauxic growth

It may be concluded from the preceding experiments that the amount of enzyme present at the end of the lag phase is sufficient to initiate growth. In order to see if the onset of growth is correlated with the enzyme content some experiments were done in which both glucose and lactose were added, in the ratio 7:3. Under these conditions the phenomenon "diauxie" (MONOD⁷) is exhibited, *i.e.* the cells grow until all glucose is consumed, after which a certain lag phase is observed before growth is resumed with lactose as energy source. The growth curve shown in Fig. 2 clearly shows this phenomenon, and it may also be noticed that the lag phase is somewhat shorter than in the experiment illustrated in Fig. 1 (about 60 rather than 90 minutes). No enzyme synthesis occurs before growth has ceased due to lack of glucose. At this time it begins almost immediately and proceeds at maximum rate after a few minutes. Already after 30 minutes the enzyme content is 1.5 times higher than at the end of the lag phase during normal adaptation, and when the growth begins it is about 5 times higher. The onset of growth is thus not directly a function of the enzyme content.

The enzyme synthesis greatly exceeds the normal content per cell, if only new

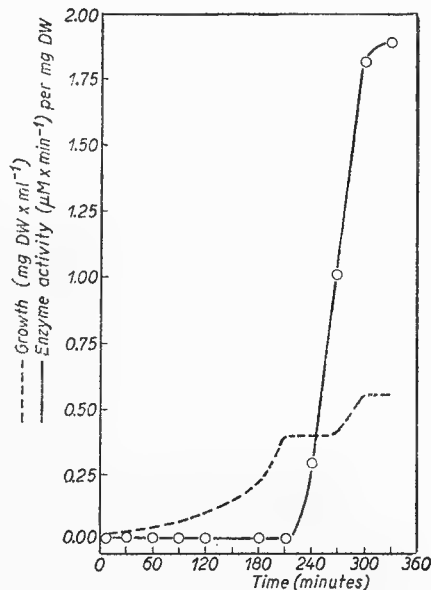


Fig. 2. Growth and induced enzyme synthesis under diauxic conditions

cells were to produce the enzyme. Thus in the interval from 270–300 minutes the synthesis amounts to $4.40 \mu\text{M} \times \text{min}^{-1}$ per mg DW. The value approached at the end of the experiment is .190, *i.e.* in the normal range.

This diauxic experiment indicates that the nutritional state of the cells influences the enzyme synthesis during the lag phase. The cells in this experiment were under optimal conditions with respect to nutrition, both because they were in the logarithmic growth phase, but also because no isolation and washing of the cells took place (*cf.* the following section).

Synthesis in N-free medium under various conditions

The preceding experiments clearly indicate that the rate of synthesis depends upon the state of nutrition of the cells. It was therefore decided to investigate the influence of this factor in the absence of nitrogen. At the same time the effect of extracting

the bacteria with distilled water was investigated.

The bacteria normally used in the present experiments were taken from cultures inoculated in the afternoon and harvested the next morning. By this time growth had ceased because of exhaustion of the energy source, glucose, and it may be presumed that also intermediate metabolites arising from glucose were more or less exhausted. The bacteria may thus be considered in a state of semi-starvation. The enzyme synthesis in these cells has been compared with synthesis in cells taken from a culture in the logarithmic growth phase.

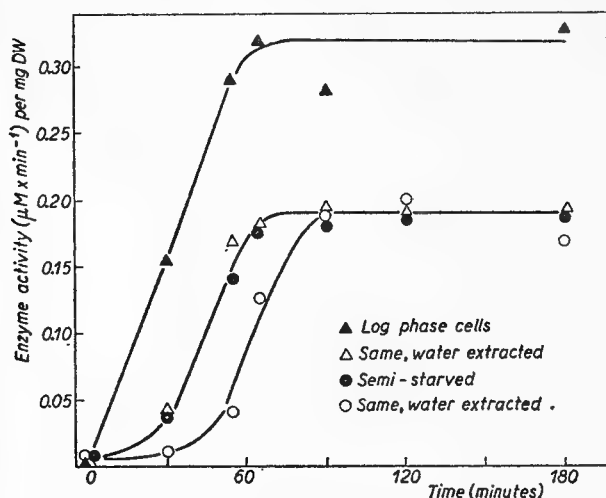


Fig. 3. Effect of starvation and extraction with water on the induced enzyme synthesis in nitrogen-free medium.

The bacteria in a "semi-starved" culture were harvested, washed in saline and suspended in N-free medium. One third of the bacteria were transferred to complete medium + glucose and allowed to grow for two hours. After harvesting, half of these bacteria, and another third of the original ones were suspended in distilled water and left at room temperature for one hour. The suspensions were shaken occasionally. All four portions were now suspended in N-free medium with lactose ($1 \text{ mg} \times \text{ml}^{-1}$) and incubated at 37°C .

The results shown in Fig. 3 demonstrate that in "semi-starved" cells both the initial rate and the extent of synthesis is lower than in the log phase cells. The maximum activity of enzyme in the latter case was about 0.32, in the former about $0.19 \mu\text{M} \times \text{min}^{-1}$ per mg DW. This latter value is about the one usually found in "semi-starved" cells. It is seen that the surplus enzyme content, as well as the higher initial rate of synthesis disappears when the cells are extracted with distilled water. This treatment thus removes factors influencing both rate and extent of synthesis, but it is apparently not possible to pass beyond the capacity possessed by the

"semi-starved" cells. This may be incidental as far as the factors responsible for the initial rate of synthesis are concerned; at least, the extraction of the "semi-starved" cells leads to a further delay in enzyme synthesis. The results seem to allow a clear distinction between two sets of factors, each responsible for one of the two aspects of enzyme synthesis discussed above.

It should be noted that the rates of synthesis, once the different initial periods are over, are constant and equal (*cf.* PORTER, HOLMES AND CROCKER¹⁰). The endogenous factors determining the apparent maximum rate under the given experimental conditions are thus very little influenced by external factors.

The influence on enzyme synthesis of adding various substances

It was shown in the preceding section that starvation and extraction of the bacteria lead to loss of two kinds of substances, some of which influence the initial rate, and some the extent of synthesis. From what is already known about induced enzyme synthesis, it is reasonable to expect that the latter substances are the nitrogen reserves from the amino acid pool, and the former substances taking part in the energy supply.

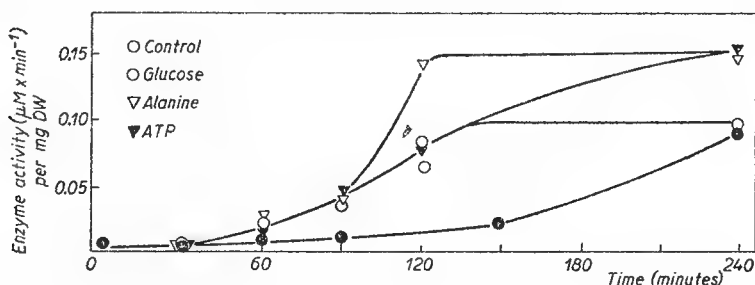


Fig. 4. Effect of addition of various substances on the induced enzyme synthesis in nitrogen-free medium.

To test this, various substances were added to bacteria which had been severely starved by keeping a "semi-starved" culture in the refrigerator for 24 hours. These bacteria were suspended in N-medium, and divided into four equal portions. To the first was added glucose ($7 \mu\text{g} \times \text{ml}^{-1}$), to the second alanine ($7 \mu\text{g} \times \text{ml}^{-1}$), to the third ATP ($35 \mu\text{g} \times \text{ml}^{-1}$), while the last served as control. In all four cases were added 1.33 mg lactose per ml . The results of enzyme determinations after various time intervals are shown in Fig. 4. The high degree of starvation is demonstrated by the enzyme synthesis in the controls, which proceeded considerably slower than in the preceding experiments. The final activity (unfortunately no level was reached) was only $0.10 \mu\text{M} \times \text{min}^{-1}$ per mg DW , *i.e.* about half the normal value. All three added substances were able to increase the rate of synthesis above that in the control, and to the same degree during the first 90 minutes. The rate of synthesis is, however, much lower than in "semi-starved" bacteria. Only in the suspension with alanine was a further increase in rate observed, but even this rate is only about $1/3$ of the normal.

The final activity reached by the bacteria supplied with glucose was the same as in the controls, but in the two other cases a higher enzyme content was found, corresponding to about $0.15 \mu\text{M} \times \text{min}^{-1}$ per mg DW .

The results are thus consistent with the suggestions above.

The effect of ATP does not necessarily suggest that adenine-N is directly used for protein synthesis, but may mean that adenine saves amino acids for building up enzyme. This suggestion conforms with the fact that a certain nucleic acid synthesis does occur under these circumstances (*cf.* LØVTRUP¹¹).

The very low rate of synthesis shows that during starvation substances are lost which are not quickly replaced, even after addition of glucose, alanine and ATP.

The diauxic experiments showed that presence of glucose prevents the induction of β -galactosidase synthesis by lactose. The above results demonstrate that when the ratio glucose/lactose is sufficiently small, here about 1/200, the presence of glucose may have an enhancing effect on the enzyme synthesis.

The effect of preceding treatment with glucose or lactose

The antagonism between glucose and lactose as far as enzyme synthesis is concerned, was further investigated in the following experiments. Bacteria from a normal "semi-starved" culture were suspended in N-free medium and divided into four equal portions. To the first two lactose was added, and to the third glucose to give a final concentration $2.4 \text{ mg} \times \text{ml}^{-1}$ while the last served as control. The bacteria

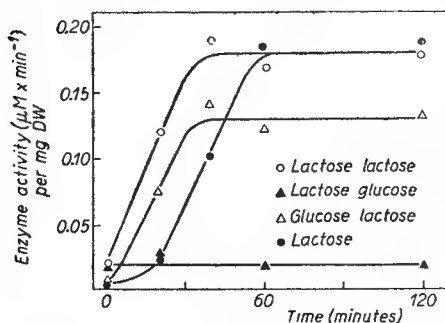


Fig. 5. Effect of preceding treatment with carbohydrates on the induced enzyme synthesis in nitrogen-free medium.

were left in the refrigerator overnight. In the morning they were harvested, washed and resuspended in N-free medium. To one of the first two portions glucose was added, to the others lactose ($1 \text{ mg} \times \text{ml}^{-1}$), and the suspensions incubated. The results of enzyme determinations after various time intervals are shown in Fig. 5.

The first thing to notice is that these bacteria, which were kept in the cold supplied with energy sources, but no nitrogen, show no signs of severe starvation contrary to those in the preceding experiment, which had available a nitrogen, but no energy source. The

control cells shows the normal course of synthesis, *i.e.* a gradually increasing rate of synthesis during the first short part of incubation, after which synthesis proceeds linearly.

In the suspensions incubated overnight with lactose, a certain increase in enzyme had occurred, from about 0.005 to $0.025 \mu\text{M} \times \text{min}^{-1}$ per mg DW. When incubated with glucose, no further enzyme synthesis occurs, but the enzyme activity remains at the high level. With lactose, synthesis starts immediately at the highest rate, but the final level is the same as in the controls ($0.19 \mu\text{M} \times \text{min}^{-1}$ per mg DW). The cells incubated with glucose also were able to begin the enzyme synthesis at maximum speed, indicating that energy supply is not a limiting factor. The final level of enzyme activity was lower, however, showing that during incubation with glucose a certain utilization of reserves had occurred.

DISCUSSION

In their paper on the kinetics of the induced synthesis of β -galactosidase in *E.coli*,

MONOD, PAPPENHEIMER AND COHEN-BAZIRE¹² discuss three possible correlations between enzyme synthesis and growth (increase in cellular mass). In all three cases it is assumed that after an initial adaptive period linearity is obtained between these two quantities, *i.e.* that the amount of enzyme protein constitutes a constant fraction of the total protein synthesized. The suggested differences are supposed to exist only during the initial phase of induced synthesis. In the simplest case, linearity will obtain throughout, and this was found by MONOD, PAPPENHEIMER AND COHEN-BAZIRE to hold under their experimental conditions. This case corresponds to that where all new, and no old cells, contain the normal amount of enzyme, although such a distinction is purely "imaginaire"¹².

The second possibility is that the rate of enzyme synthesis gradually increases, showing an "autocatalytic" course. Adaptive growth, or enzyme synthesis has often been found to exhibit such "autocatalysis", and various hypotheses have been advanced, trying to explain the mechanism of adaptation on this basis. However, it seems certain by now that induced enzyme synthesis is not autocatalytic, *i.e.* the enzyme proper does not in any way influence the synthesis of more enzyme of its own kind¹². The gradual increase in enzyme synthesis is caused by lack of various limiting factors, when these are supplied enzyme synthesis proceeds at maximum rate almost instantaneously (PORTER, HOLMES AND CROCKER¹⁰, *cf.* also below). According to the third possibility enzyme synthesis begins at a very high initial rate, which gradually decreases until linearity between growth and enzyme synthesis is reached. This mechanism should obtain, if the enzyme is formed by conversion of a specific precursor. The precursor hypothesis seems to be ruled out also. Both the experiments of HALVORSON AND SPIEGELMAN¹³ on yeast, and those of MONOD, PAPPENHEIMER AND COHEN-BAZIRE¹² on *E.coli* seem to demonstrate that the induced enzyme synthesis is a *de novo* protein synthesis. However, as shown in the present paper, a rather extensive synthesis may occur even when no growth occurs, and when growth is allowed, a considerable surplus enzyme synthesis occurs (*cf.* also the diauxic experiment). These results thus correspond to the third possibility mentioned above, without therefore, of course, constituting a proof of the precursor theory.

The results are on the whole compatible with the assumption that during induction both old and new cells acquire the enzyme. In order to explain the difference between these results and those obtained by MONOD, PAPPENHEIMER AND COHEN-BAZIRE the attention should be directed towards one important difference in the experimental conditions. In the latter case there was always an energy source (maltose or succinate) present in the medium besides the inductor (*e.g.* lactose, melibiose or some β -galactoside) which in most cases could not be used for energy supply. In the present experiments the inductor, lactose, was the only available energy source. This fact seems to be the most reasonable explanation of the observed difference. It might also be mentioned that no synthesis occurred under the experimental conditions employed by MONOD, PAPPENHEIMER AND COHEN-BAZIRE, when the cells were starved, either with respect to the energy, nitrogen or sulfur source. Whatever the explanation of the differences may be, the fact remains that induced enzyme synthesis may occur at a rate above that indicated by the "normal" linear relation between enzyme formation and growth (*cf.* the following paper¹¹, in which isotopes have been used to analyze this phase of synthetic activity in more detail).

As mentioned above, it was found by PORTER, HOLMES AND CROCKER¹⁰ that the rate of induced synthesis of β -galactosidase is linear when all limiting factors have been removed, and the same has been found in other cases of induction (*cf.*¹⁰). The present results extend the previous ones by showing the same to hold even when synthesis occurs in the absence of any exogenous nitrogen source. It was found that the rate-limiting factors in N-free medium apparently are involved in the supply of energy, as in cells in the logarithmic growth phase, or pre-incubated in either glucose or lactose, the induced synthesis proceeds linearly from the beginning. Extraction with distilled water, or starvation with respect to energy sources, lead to losses in these factors, as reflected by a decrease in the initial rate of enzyme synthesis, the kinetics of which thus exhibits an "autocatalytic" course. After prolonged starvation a condition is reached where the addition of small amounts of energy sources like glucose, alanine and ATP are unable to restore the rate of synthesis, although a certain improvement occurs.

Concerning the endogenous nitrogen reserves available for enzyme synthesis, cells in the logarithmic growth phase were found to contain more than other cells, judging from the extent of enzyme synthesis. However, the surplus might be extracted with water, whereas the final enzyme content in "semi-starved" cells was uninfluenced by extraction. Incubation with glucose, as well as prolonged starvation lead to partial exhaustion of the nitrogen reserves, as indicated by a reduced level of synthesis. The addition of nitrogen sources, such as alanine and even ATP, to severely starved cells increases the final level of enzyme synthesis, although no complete restoration was found.

SUMMARY

In *E. coli* the content of β -galactosidase in already adapted cells corresponds to a hydrolysis of about $2 \mu\text{M ONPG} \times \text{min}^{-1}$ per mg dry weight. In adapting cells the enzyme is synthesized during the lag phase, and during the early growth phase the amount of enzyme synthesized per mg dry weight of new cells considerably exceeds the value mentioned above.

By growing the bacteria in the presence of both glucose and lactose (diauxie), it was shown that the time of growth initiation is not solely a function of the amount of enzyme present in the cells.

Induction of β -galactosidase synthesis occurs even in the absence of any exogenous nitrogen source, at least when lactose is the only energy source. Under these conditions the extent of enzyme synthesis may be decreased by starvation, by extraction with water and by pretreatment with glucose. However, the synthetic capacity may be recovered, partly at least, by addition of nitrogen-containing substances such as alanine and ATP.

The initial rate of enzyme synthesis in N-free medium is also reduced by starvation and by extraction with water. Addition of small amounts of glucose, alanine or ATP, as well as pre-treatment with glucose or lactose increases this rate.

RÉSUMÉ

Chez *E. coli* B, la teneur en β -galactosidase des cellules déjà adaptées correspond à l'hydrolyse d'environ $2 \mu\text{M ONPG} \times \text{min}^{-1}$ par mg de poids sec. Dans les cellules en voie d'adaptation l'enzyme est synthétisé pendant la phase de latence. Pendant la phase initiale de la croissance la quantité d'enzyme synthétisé par mg de poids sec de cellules nouvelles surpasse considérablement le taux mentionné ci-dessus.

En cultivant les cellules sur glucose et sur lactose à la fois (diauxie), nous avons constaté que le début de la croissance n'est pas simplement une fonction de la quantité d'enzyme induit présent dans les bactéries.

L'induction de la synthèse de β -galactosidase peut avoir lieu même en l'absence complète d'une source exogène d'azote, du moins si le lactose est la seule source d'énergie. Sous ces con-

ditions, on peut diminuer la concentration de l'enzyme induit par carence, par extraction aqueuse et par traitement préliminaire au glucose. La capacité de synthèse peut être régénérée, partiellement au moins, par l'addition de substances contenant de l'azote comme p. ex. l'alanine et l'ATP.

La vitesse initiale de la synthèse de l'enzyme dans le milieu exempt d'azote peut être diminuée elle aussi par carence ou par extraction aqueuse. L'addition de petites quantités de glucose, d'alanine ou d'ATP, ainsi qu'un traitement préliminaire au glucose ou au lactose augmentent cette vitesse.

ZUSAMMENFASSUNG

Bei *E. coli* B entspricht der β -Galaktosidase-Gehalt in den bereits adaptierten Zellen der Hydrolyse von ca. $2 \mu\text{M ONPG} \times \text{min}^{-1}$ pro mg Trockengewicht. In den sich adaptierenden Zellen wird das Enzym während der Latenzphase synthetisiert, und während der frühen Wachstumsphase überschreitet die pro mg Trockengewicht neuer Zellen synthetisierte Enzymmenge diesen Wert beträchtlich.

Beim Züchten der Bakterien sowohl auf Glukose wie auf Laktose (Diauxie) wurde festgestellt, dass der Zeitpunkt des Wachstumsbeginns nicht einfach eine Funktion der in den Zellen vorhandenen Enzymmenge darstellt. Die Induktion der Synthese von β -Galaktosidase findet ebenfalls in N-freiem Medium statt, wenigstens wenn Laktose die einzige Energiequelle ist. Unter diesen Bedingungen kann die Konzentration des induzierten Enzyms durch Aus Hungern, durch Wasserextraktion und durch Vorbehandlung mit Glukose vermindert werden. Die Synthesekapazität wird aber wenigstens teilweise durch Zugabe von stickstoffhaltigen Substanzen wie Alanin und ATP regeneriert.

Die Anfangsgeschwindigkeit der Enzymsynthese in N-freiem Medium wird ebenfalls durch Aus Hungern und durch Wasserextraktion vermindert. Die Zugabe von kleinen Mengen Glukose, Alanin oder ATP, sowie die Vorbehandlung mit Glukose oder Laktose vergrößern diese Geschwindigkeit.

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IV.C.4 The Induced Synthesis of β -Galactosidase in *E. coli*, 2, Analysis of the Accompanying Synthetic Activity by Means of Isotopes

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INTRODUCTION

In the preceding paper¹ it was shown that when the synthesis of β -galactosidase is induced by lactose in *E. coli*, the increase in enzyme activity exceeds that to be expected from the increase of cells, but corresponds rather well to the assumption that both new and old cells acquire the enzyme. In nitrogen-free medium the addition of lactose stimulates a synthesis of the enzyme at the cost of the nitrogen reserves in the cells.

Among the many questions raised by these findings several would appear answerable by determining the incorporation of radioactive isotopes associated with the induced enzyme synthesis. An attempt has been made in the present paper to answer a few of the problems which might be solved in this way.

MATERIAL AND METHODS

Biological material. The experiments were performed on *E. coli* B. Regarding the details of culture technique the reader may refer to ROBERTS *et al.*².

Enzyme determinations. The methods used for extraction and determination of the enzyme were the same as described in the preceding paper¹.

Isotope technique. Samples for determination of uptake of isotopes were centrifuged, washed in saline, and centrifuged again. In the experiments with labelled sulfate the cells were first extracted with 5 ml 5% cold TCA to remove acid-soluble sulfur compounds (*cf.*²). The residue, assumed to contain only protein-bound S, was dissolved in 5 ml 0.1 N NaOH. Samples of 0.5-1 ml were taken directly for counting. In the experiments with ¹⁴CO₂ the sample for each time interval had to be in a separate, closed bottle. Normal aeration was thus not possible and the bottles were therefore shaken in an air thermostat at 37° C. The samples taken for counting were extracted with cold TCA, alcohol, alcohol-ether (1:1), and ether in succession, 5 ml of each liquid. The nucleic acids were extracted by hot 5% TCA (100° C for 20 minutes). The residue, considered to be protein, was dissolved in 5 ml 0.1 N NaOH. Samples generally varying between 0.5-1 ml were taken from the two latter fractions for counting (*cf.*²).

RESULTS

Experiments with ^{35}S *Enzyme and protein synthesis in complete medium*

In the first experiment to be reported a comparison was made between increase in dry weight (DW) and uptake of sulfate in the protein fraction in cells growing in glucose and in lactose, respectively. The bacteria were grown in a medium containing $14 \mu\text{g S} \times \text{ml}^{-1}$, *i.e.* about half the normal concentration, radioactive sulfate ($447 \text{ counts} \times \text{sec}^{-1} \times \text{ml}^{-1}$ or $32 \text{ counts} \times \text{sec}^{-1}$ per mg S) and either glucose or lactose ($1 \text{ mg} \times \text{ml}^{-1}$). Samples for counting and density determinations were taken at regular intervals, and in the lactose experiments extra samples were taken for enzyme determinations.

The two growth curves are shown in Fig. 1, where also the increase in DW has been plotted against the uptake of protein S. It is seen that there is a linear relationship between these two quantities, identical within the limits of error for glucose and lactose-grown bacteria, and corresponding to $5 \mu\text{g}$ protein-S per mg DW . When the lag phase is over, the sole difference between the bacteria in the two cultures presumably is the synthesis of β -galactosidase. The result obtained thus leads to the hardly surprising conclusion that the amount of this enzyme is negligible compared to the total protein content of the bacteria.

By calculating the ratio between enzyme activity and incorporation of sulfur (enzyme and protein synthesis) it is possible to estimate whether the synthetic activity during the lag phase is quantitatively different from that occurring after adaptation has occurred, *i.e.* during growth proper. The results of such calculations from two different experiments are shown in Fig. 2, from which it is seen that during the lag and early growth phase the ratio between these two quantities is higher than later on; the highest value measured being about seven times the final value. If as concluded above

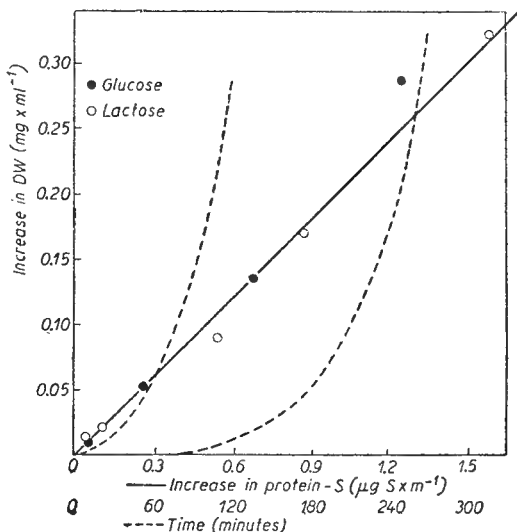


Fig. 1. Growth and increase in protein-S under normal conditions and during adaptation.

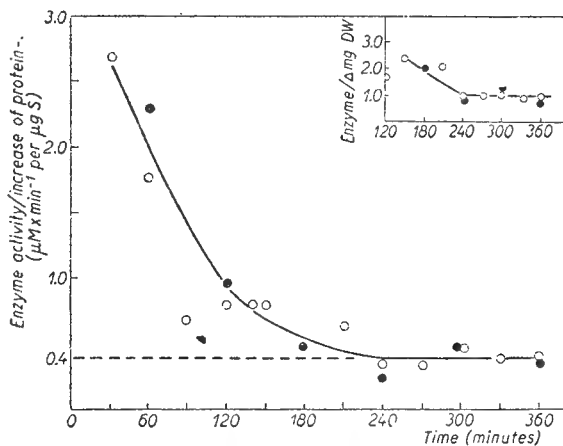


Fig. 2. Changes in the relative enzyme synthesis in complete medium. The two different symbols indicate two different experiments.

the amount of enzyme is negligible compared to total protein, we may thus conclude that other proteins than the enzyme proper are synthesized during the lag phase, although the production of β -galactosidase enjoys a certain preference. This result is in conformance with the observation reported in the preceding paper, *viz.* that the amount of enzyme synthesized under such experimental conditions exceeds the amount to be expected from the increase in cell number (DW), *cf.* the graph inserted in Fig. 2, which shows the relative ratio between total enzyme and increase in DW. The two curves are very similar, but the latter cannot be traced back as far as the former, because no measurable growth occurred during the first 120 minutes.

Enzyme and protein synthesis in N-free medium

The bacteria used in the present experiments were starved to get as great effects as possible. The starvation was achieved by harvesting and washing the bacteria from a culture, and keeping the resulting pellet in the refrigerator for about two weeks. As will be seen from the experiments, the degree of starvation obtained under these conditions (minimum of liquid, no nutrient solution) during this long period is comparable with that reached in the preceding paper by keeping a glucose-exhausted culture in the refrigerator overnight.

The bacteria were suspended in 35 ml N-free medium. One portion of 3 ml was transferred to complete medium, containing 2.5 mg glucose per ml, and incubated for two hours. A sample of 10 ml was treated similarly, with the exception that N-free

medium was used. A second portion of 10 ml was centrifuged and suspended in distilled water for one hour, while a third 10 ml sample was left as control. After the treatment the cells were centrifuged, washed with saline, and each portion added to N-free medium, containing 1 mg lactose and 7 μ g S per ml. Radioactive sulfate was also added, the initial content being 637 counts \times sec⁻¹ \times ml⁻¹; *i.e.* 91 counts \times sec⁻¹ per μ g S. Samples for determination of enzyme activity and uptake of sulfate were taken during the following 3 hours. The results are shown in Fig. 3.

The cells incubated in complete medium show a very high initial rate of enzyme synthesis (although a slight lag is seen, *cf.* 1), and the final activity (0.40 μ M \times min⁻¹ per mg DW) is twice the value for "semi-starved" cells. The enzyme synthesis ceases after 60 minutes, but the uptake of sulfur in the protein

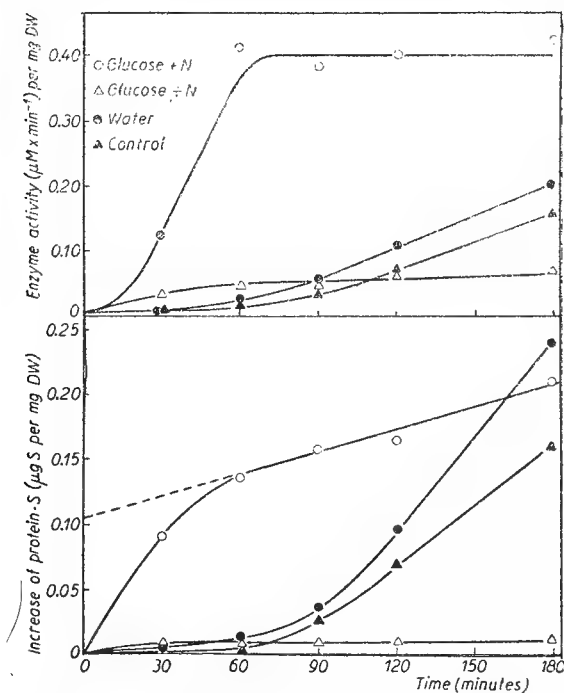


Fig. 3. Effect of preceding incubation with glucose in normal and in nitrogen-free medium and of extraction with water on the induced enzyme synthesis and on the incorporation of protein-S in nitrogen-free medium.

fraction, which is rapid while enzyme is produced, continues slowly throughout the experiment at a rate of $5.5 \cdot 10^{-4} \mu\text{g S} \times \text{min}^{-1} \text{ per mg DW}$.

The initial rate of both enzyme and protein synthesis are relatively high in the bacteria preincubated with glucose in N-free medium. This indicates, in agreement with the results in the preceding paper, that the supply of energy, improved by the incubation in glucose, determines the rate at which synthesis begins. Both the increase in enzyme activity and the uptake of sulfate cease almost completely after 30 minutes, the final enzyme level corresponding to $0.05 \mu\text{M} \times \text{min}^{-1} \text{ per mg DW}$, *i.e.* about 25 % cent of normal. This low enzyme content partly reflects the degree of starvation, but may also be due to losses in the nitrogen reserves as a result of the preincubation with glucose (*cf.* the experiments with $^{14}\text{CO}_2$ -uptake).

The considerable decrease in initial rate of enzyme synthesis resulting from starvation (*cf.* 1) is exhibited both by the controls and the water extracted bacteria. The same phenomenon is also shown by the curves for uptake of protein-S. These two groups run almost parallel, although the water extracted cells seem to do rather better than the controls. The enzyme activity in these cells is after 180 minutes close to the normal maximum content in "semi-starved" cells, when incubated in N-free medium, and it exceeds by far the amount of enzyme in the cells preincubated with glucose in N-free medium. When the uptake of protein-S is compared, it is seen that it approaches, and for the water-extracted cells even exceeds, that occurring in the bacteria in the first group, which were taken in the logarithmic growth phase. The source of nitrogen sustaining this extensive synthesis seems rather mysterious, but the most reasonable assumption is that it comes from autolyzing cells. In that case the enhancing effect of the water treatment might be easier understandable. To bring agreement between the results it seems necessary to assume that incubation of the exhausted cells with glucose reduces the degree of autolysis.

Calculating the ratio between enzyme activity and incorporation of protein-S may also in this case give a measure of the "efficiency" of enzyme synthesis. In doing these calculations, it seems reasonable to correct the values in the first group for the uptake of S occurring after the enzyme synthesis has ceased. The results of the calculations are shown in Fig. 4. The variations are seen to be great, but even so it seems clear that there is a quantitative difference between the glucose-incubated cells on one hand, and the water extracted and the controls on the other. The enzyme synthesis which is of very short duration in the two first groups, is highly "efficient", corresponding to about $4\text{--}6 \mu\text{M} \times \text{min}^{-1} \text{ per } \mu\text{g protein-S}$, as compared to 0.4 for adapted growing cells (*cf.* Fig. 2). In the two other groups, in which synthesis goes on for a longer period, the "efficiency" seems rather high in the beginning, but after 120 minutes a level around $1 \mu\text{M} \times \text{min}^{-1} \text{ per mg protein-S}$ has been reached. This decrease in "efficiency" is thus completely analogous to that occurring in complete medium (*cf.* Fig. 2), but the "efficiency" is somewhat higher after 180 minutes in N-free medium.

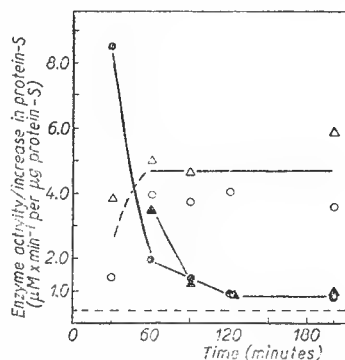


Fig. 4. Changes in the relative enzyme synthesis in nitrogen-free medium. The signification of the different symbols is the same as in Fig. 3.

Experiments with ¹⁴C

Uptake of CO₂ in growing, adapting and resting cells

Normal "semi-starved" bacteria were harvested, washed in saline and resuspended in 350 ml nitrogen-free medium, boiled to remove CO₂. The experiment comprised three groups of four bottles. In the first group was added 40 ml bacterial suspension and 1 ml 10% lactose to each bottle, in the second group lactose was replaced by glucose, and in the third group was added 5 ml bacterial suspension, 35 ml complete medium and 1 ml glucose. To each bottle was further added ¹⁴CO₂ (30 μl of a solution containing about 25 millicurie per ml). After varying time intervals bottles were taken out for determination of DW (optical density) and uptake of CO₂. The DW in the first groups was 0.70 ± 0.03 mg DW per ml, in the third group DW increased from 0.11 to 0.54 mg per ml. The results of the countings are shown in Table I.

TABLE I
Uptake of ¹⁴CO₂ (counts × sec⁻¹ × ml⁻¹)

Time (minutes)	Lactose		Glucose		Glucose + nitrogen	
	Nucl. acids	Protein	Nucl. acids	Protein	Nucl. acids	Protein
30	0.10	0.17	0.12	0.62	1.84	4.22
60	0.08	0.22	0.12	0.66	4.83	7.92
120	0.34	1.16	0.15	0.92	10.1	17.6
180	0.65	2.58	0.20	1.01	11.3	20.1
<hr/>						
Protein Nucl. acid	(30 + 60 + 120) 3.0		5.6		1.7	

In the first group a slight gradual increase occurs in the rate of uptake. The final uptake in the nucleic acid and the protein fractions are 3 and 2.5 times higher, respectively, than in the second fraction. In this a very rapid uptake occurs during the first 30 minutes, after which the uptake continues at a very low rate. In the third group the rate of incorporation is very high, continuing linearly during the first two hours, after which a decrease occurs (isotope dilution?). The results clearly demonstrate that both nucleic acids and proteins are synthesized. One point of interest in this connection is to determine the ratio between protein and nucleic acid synthesis. The value of this ratio may be obtained from the results in Table I. Using only the three first values, because of the lack of linearity after 180 minutes, we get 3.0, 5.6 and 1.7 respectively for the three groups. The value for the third group may be considered quite accurate, because of the linearity and the high counting values, and the value for the first group has been confirmed by results to be reported below. The value for the second group is higher than for the first group in the present experiments, but this has not been confirmed. It would seem warranted to conclude from these results that the specific adaptive metabolism is characterized by a relatively high protein synthesis.

The second experiment to be reported was similar to the one first described, with the exception that nitrogen-free-medium was used in all three groups, and that no carbohydrate was added to the last group. Furthermore the amount of ¹⁴CO₂ was twice as high as in the preceding experiments.

It is seen in Fig. 5 that the very rapid initial uptake in the presence of glucose has been confirmed, but after 60 minutes only a very slight further uptake occurs. In the medium containing no carbohydrate there is a very slow uptake during the first three hours, but after this time no further uptake occurs. The final values are about half of those in the glucose medium. The initial rate of uptake is much lower in lactose than in glucose, but the uptake continues during the whole experimental period, and the uptake after 300 minutes is about 3 times higher in both fractions. The protein/nucleic acid ratio is the same in all three cases, taking the values at 180 minutes we get 3.3, 3.5, and 3.2 respectively.

These results thus show that the presence of glucose allows an uptake of CO_2 in the protein and nucleic acid fractions over and above that occurring in the absence of any carbohydrate. Moreover, in the presence of lactose this process goes even further. It seems permissible to conclude that the addition of lactose mobilizes reserves which are not normally available. Thus lactose apparently may both initiate the synthesis of β -galactosidase and the mobilization of nitrogen reserves. The uptake of CO_2 apparently continues longer than the normal duration of adaptive enzyme synthesis. Unfortunately no enzyme determinations were made to check this point.

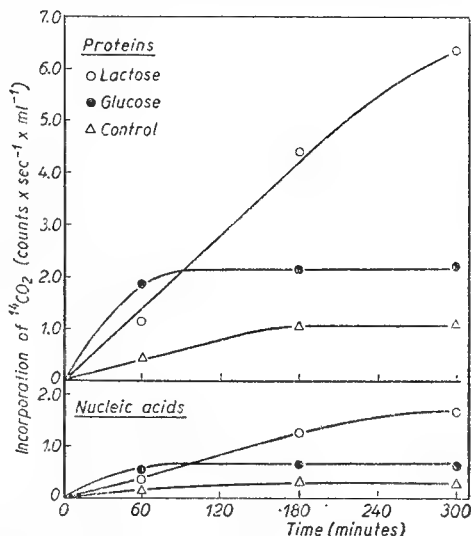


Fig. 5. Incorporation of $^{14}\text{CO}_2$ in the protein and nucleic acid fractions in nitrogen-free medium before and after addition of lactose and glucose, respectively.

DISCUSSION

It is unquestionable that the clue to a deeper understanding of the mechanism of induced synthesis of enzymes is the application of isotopes. A promising beginning has been made by HOGNESS, COHN AND MONOD³ in a study of the precursors and turnover of β -galactosidase in *E.coli*. The results presented here deal with another aspect of the synthetic mechanism, but should be regarded merely as an orientation.

A few definite results have been obtained, however, illustrating the peculiarities of adaptive metabolism. It was reported in the preceding paper that when induction of β -galactosidase synthesis occurs in *E.coli* with lactose as the only energy source, a certain surplus synthesis of enzyme occurs during the early growth period, besides that occurring in the lag phase. This means that the ratio between enzyme synthesis and increase in DW decreases with time. Using radioactive sulfate it has been demonstrated that the ratio between enzyme and protein synthesis (enzyme activity/uptake of protein-S) behaves similarly. This ratio may be determined even before any measurable growth occurs. Values approaching seven times the normal for this ratio have been attained after 30 minutes incubation. Measuring the same ratio when adaptation occurs in the absence of nitrogen, still higher values (15–20 times

normal) were found. These results thus indicate that metabolism during the initial phase of adaptation is specifically oriented towards synthesis of the particular enzyme.

With radioactive CO_2 it was possible to demonstrate that the ratio between protein and nucleic acid synthesis is twice as high during adaptation in the absence of nitrogen than during normal growth. This finding further stresses the special character of the processes during the initial phase of induced synthesis. Another result obtained by the work with $^{14}\text{CO}_2$ is that in the absence of nitrogen the uptake of CO_2 in (synthesis of) proteins and nucleic acids is considerably higher with lactose than with glucose. It thus seems that during the induced synthesis nitrogen reserves are mobilized which are not normally accessible.

ACKNOWLEDGEMENTS

The author gratefully acknowledges the assistance, advice and encouragement given by all the members of the staff during his visit at the Biophysics Laboratory, Carnegie Institution of Washington.

SUMMARY

By following simultaneously the uptake of ^{35}S and enzyme synthesis in *E. coli* B during adaptation to lactose it was found that the ratio: Enzyme synthesis/protein synthesis is much higher during the earlier phases of adaptation than later on.

Using $^{14}\text{CO}_2$ it could be shown that the ratio: Protein synthesis/nucleic acid synthesis is higher in adapting than in growing cells. These results thus demonstrate that synthetic activity during adaptation is quantitatively different from that occurring during normal growth.

RÉSUMÉ

Chez *E. coli* B on a déterminé simultanément l'incorporation de ^{35}S et la synthèse induite de β -galactosidase pendant l'adaptation sur lactose. On a trouvé ainsi que pendant la phase de latence et au début de la croissance, le taux de la relation: Synthèse enzymatique/synthèse protéinique était considérablement augmenté.

En utilisant $^{14}\text{CO}_2$ on a trouvé que le taux de la relation: Synthèse protéinique/synthèse acide nucléique était plus haut pendant l'adaptation que pendant la croissance normale.

Ces résultats montrent que l'activité synthétique pendant l'adaptation (induction) se distingue quantitativement de celle pendant la croissance.

ZUSAMMENFASSUNG

Bei *E. coli* B wurde gleichzeitig die Aufnahme von ^{35}S und die induzierte Synthese von β -Galaktosidase während der Adaptation auf Laktose beobachtet. Es wurde dadurch gefunden, dass die Verhältniszahl: Enzymsynthese/Proteinsynthese während der Latenzphase und während der frühen Wachstumsphase beträchtlich erhöht ist.

Durch Anwendung von $^{14}\text{CO}_2$ konnte nachgewiesen werden, dass die Verhältniszahl: Proteinsynthese/Nukleinsäuresynthese während der Adaptation höher ist als während des Wachstums.

Diese Ergebnisse zeigen, dass die synthetische Aktivität während der Adaptation (Induktion) quantitativ verschieden ist von derjenigen, die während des normalen Wachstums besteht.

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- ³ D. S. HOGNESS, M. COHN AND J. MONOD, *Biochim. Biophys. Acta*, 16 (1955) 99.

Comment. These papers lost their timeliness in the four years between the completion of the work and their publication. Richard B. Roberts.

IV.C.5 Osmotic Upshock

(Reprinted from Carnegie Institution of Washington Year Book 56, p. 124, 1957.)

R. B. Roberts

A sudden increase of the osmotic strength of the medium causes a quite different effect from a sudden decrease. When a growing cell suspension is suddenly mixed with an equal volume of medium containing 1 *M* sucrose, synthesis of nucleic acid and protein stops. After a period of roughly 8 minutes, the synthetic activities resume; during this period the metabolic pool materials continue to be incorporated. Similar effects are observed with glucose and sodium chloride, but proline causes no major effect.

If the cells are washed with Tris medium just after sucrose is added, there is no loss of P^{32} -labeled pool materials. At the end of 8 minutes a Tris wash removes

half the pool. It appears that the sucrose penetrates slowly into some structure of the cell associated with holding the P^{32} -labeled pool. Before the sucrose has penetrated, the structure is perhaps dehydrated, causing the cessation of synthesis. After the sucrose has penetrated, the structure can be osmotically shocked by washing the cells with Tris. It is striking that the period for this action is quite different from the period required for sucrose to penetrate the cell wall of lysozyme-treated cells in the procedure for protoplast formation. It may also be significant that glucose has the same action, whereas it is less effective in the protoplast procedure.

Comment. These experiments have a continuing interest, as they indicate the sensitivity of macromolecular synthesis to physical disturbances to intracellular organization. Richard B. Roberts.

IV.C.6 The Formation of Protomorphs

(Reprinted, by permission, from Microsomal Particles and Protein Synthesis, pp. 151-155, Pergamon Press, New York, 1958.)

Frank T. McClure and Richard B. Roberts

Applied Physics Laboratory, The Johns Hopkins University,
Silver Spring, Maryland, and Department of Terrestrial Magnetism,
Carnegie Institution of Washington, Washington, D. C.

Disrupted cells of *Escherichia coli* were suspended in a number of different solutions to find which ones were suitable for making stable preparations of ribosomes. Among those tested were some containing manganese and magnesium, because these ions had been found essential for incorporation of amino acids by cell-free systems [1, 2]. After standing for several hours these solutions became turbid and finally gave a white precipitate of unusual appearance.

Examination of these solutions in the phase contrast microscope showed that the turbidity was caused almost entirely by the presence of large numbers of nearly spherical, highly refractile particles with diameters of 1 to 5 microns (figs. 1 and 2).

The appearance of these cell-like particles in a solution that originally contained nothing visible in the microscope was quite surprising. The formation of large, stable aggregates with distinct boundaries from a fluid containing macromolecules in a homogeneous suspension seemed to illustrate a process which perhaps was important in the origin of life. Accordingly, we proceeded to investigate some of their properties. It was soon found that the particles contained protein, nucleic acid, and lipid in proportions typical of biological materials. Because they are formed from protoplasm and have distinctive shape we refer to them as "protomorphs" to distinguish them from other particles or structures that exist in the living cell.

The usual procedure for preparation of protomorphs is as follows: Harvest 10 g wet weight of *Escherichia coli* cells growing in synthetic medium "C" [3]. Wash twice with tris(hydroxymethyl)aminomethane-succinate buffer, 0.01 M,

¹ Present address: Johns Hopkins University, Applied Physics Laboratory, 8121 Georgia Avenue, Silver Spring, Maryland.



Fig. 1. Protomorphs in the solution from which they form.

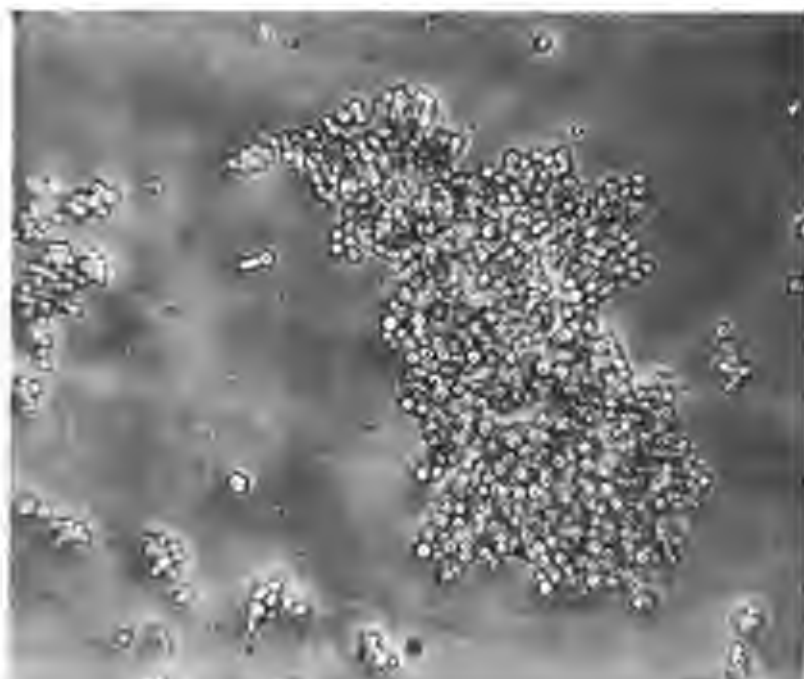


Fig. 2. Aggregated protomorphs scraped off glass surface. Phase contrast photomicrographs taken by W. R. Duryee.

pH 7.6 (TS). Suspend with 10 ml TS, and break the cells by forcing the suspension through an orifice at 10,000 psi with a flow rate of roughly 2 ml/min. Add TS to bring the total volume to 50 ml, and centrifuge 15 minutes at 40,000*g*. Discard the precipitate, which contains unbroken cells and large fragments of cell walls. Centrifuge again at 105,000*g* for 15 minutes, and discard the precipitate, which contains smaller fragments of cell walls and a small proportion of the ribosomes. Dilute the supernatant fluid with TS to 100 ml, and add MgCl_2 and MnCl_2 to make it 0.005 *M* in each. In 5 to 48 hours the solution will show turbidity because of protomorph formation. The entire procedure is carried out at 0° to 5° C.

The yield of this procedure is variable, as is the time required for formation of the protomorphs. Some of the sources of variability have been identified; others remain unknown.

The addition of manganese is essential. No protomorphs were formed when Mn^{++} was omitted even though adequate Mg^{++} was present. Higher concentrations of Mn^{++} (0.01 *M* to 0.5 *M*) caused the formation of a precipitate of particles of irregular shape and widely variable size. No attempt was made to find other cations that might substitute for the Mn^{++} . The Mg^{++} was not essential but seemed to increase the yield. No difference was noted whether the magnesium was added before or after the cells were broken; thus there would appear to be no difference to protomorph formation whether the ribosomes were in the 80 S form or not (see paper 3 of this volume).

No protomorphs were formed when the *pH* of the solution was outside the limits 7 to 8; a *pH* of 7.5 seemed to be optimum.

The concentration of orthophosphate affects the yield. When the cells are carefully washed in TS before breaking, the phosphate of the growth medium is removed and phosphate must be added to give a concentration of 10^{-3} *M*. If higher quantities of phosphate are added the particles become less refractile in appearance and are dark, rough, and "hairy." At still higher concentrations of phosphate, precipitates are formed in manganese solutions lacking cellular material. Although these inorganic precipitates have only a slight resemblance to the protomorphs, it is possible that the inorganic material provides a framework on which the organic material deposits.

The concentration of the cellular material is important. When the usual procedure was followed a twofold dilution of the cell extract prevented the formation of protomorphs. The presence of cell-wall material was not important to the yield; the yield was the same whether or not the centrifugation steps to remove cell walls and unbroken cells were omitted. When the wall material was present the protomorphs appeared less smooth, as if irregular fragments of wall had been incorporated.

The constituent responsible for the sensitivity to the concentration is probably deoxyribonucleic acid (DNA). The addition of DNAase invariably prevents the formation of protomorphs. The pressure cell routinely used to disrupt the cells also degrades DNA, as preparations of DNA lose their viscosity

on being forced through the orifice of the pressure cell. Cell juices prepared with the pressure cell do not show the peak characteristic of DNA in their sedimentation diagram [4]. It is these preparations (made with the pressure cell) that will not give protomorphs when diluted. In contrast, preparations made by grinding the cells with alumina or by lysozyme treatment followed by osmotic shock (methods which preserve DNA) do yield protomorphs even at one-tenth the usual concentration. Finally, the addition of DNA to dilute pressure-cell preparations restores the yield. It seems quite likely that variations in the pressure and in the conditions at the orifice during the disruption of the cells affect the quantity of intact DNA remaining and thereby influence the yield in an erratic way.

The formation of protomorphs was photographed by Drs. B. Hoyer and N. Kramis, of the Rocky Mountain Laboratory, U. S. Public Health Service. Their time-lapse photomicrography shows that the growth of an individual protomorph from its first appearance to full size requires only a few minutes after a much longer induction period. There may well be a slow process of nucleation followed by a rapid process of growth. Neither fission nor fusion played any part in the growth process.

Once formed, the protomorphs are stable. Unlike simple coacervate particles which exist only in a narrow *pH* range and have a strong tendency to fuse or to dissolve, protomorphs can be handled like bacteria or yeast. There is no difficulty in centrifuging the photomorphs into a pellet (1000g) and resuspending in fresh media. They are quite stable in a number of ordinary media, and persist for weeks even though overgrown by bacterial contamination. They are not dissolved by short exposures to ammonia (1 *M*), 5 per cent trichloroacetic acid (TCA), ethanol, or ether. They are dissolved in 0.01 *M* ethylenediaminetetraacetic acid to give a clear solution.

On standing, glass vessels containing protomorph suspensions develop a white film over the surface. Microscopic examination of the material scraped from the glass indicates that the protomorphs have formed a moderately well packed monolayer on the surface (fig. 2).

The organic components of the particles had roughly the proportions found in living tissues. They contain ultraviolet-absorbing material which hydrolyzes to yield the bases expected from ribonucleic acid. In addition the diphenylamine test [5] indicates that a small part (10 per cent) of the ultraviolet absorption is due to DNA. The ratio of nucleic acid to protein (measured by the Folin reaction [6]) is 1/6 as compared to 1/4 in the bacterial juice. Paper chromatography shows the presence of lipid material.

Incorporation experiments were carried out with thoroughly washed preparations of protomorphs. Radioactive phosphate was incorporated at a constant, high rate for several hours. This process was not studied in any detail because all the radioactivity so incorporated could be extracted with cold TCA and there was no evidence of incorporation into macromolecules.

The incorporation of radioactive amino acids was of more interest. The re-

sults were erratic from one preparation to another, and the incorporation rate was only 1/1000 that of intact cells at the highest. Accordingly contamination by intact cells was a constant worry. The number of intact cells was estimated both by plate counts and by microscopic examination. The uptake observed was as much as 100 times that which could be attributed to the contaminants.

There were also several qualitative features which distinguish the behavior of the protomorph preparation from that of intact cells. In the first place, the incorporation doubled if ATP was added or if the concentration of the amino acid mixture was doubled. These variations have little effect on incorporation by whole cells. Secondly, the distribution of incorporated radioactivity among the fractions soluble in cold TCA, alcohol, ether, and hot TCA was different from that obtained with whole cells [3]. Finally, the hot-TCA-insoluble material after hydrolysis yielded a pattern of radioactive amino acids different from the mixture supplied and different from what would be incorporated by whole cells.

Accordingly we believe that the observed incorporation was in fact real, though not reproducible from day to day. Since these experiments were done we have learned of the activity of the cell-wall fraction in protein synthesis [7, 8]. In retrospect it seems quite likely that the variability in the synthetic capacity of the protomorphs may have been due to a variability in their content of cell-wall fragments.

These protomorphs are of course very different from the ribosomes which are the subject of this symposium, but there may be a relationship between them. It is a common belief that the bacterial cell is not a homogeneous mixture of its various components; on the contrary, various lines of evidence indicate that it has a high degree of organization. Organization in turn implies the action of forces between the various constituents such as DNA, RNA, protein, and ribosomes. It is possible that the aggregation of these cellular constituents into protomorphs may be another manifestation of the same forces which maintain organization in the living cell and may furnish a material in which the forces are more amenable to study. If so, the protomorphs may eventually contribute to our knowledge of how the ribosomes are organized within the cell.

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Comment. These experiments were a fascinating diversion. Early hopes that these particles replicated a structure important for protein synthesis rapidly faded. Year Book 57 reports:

"In last year's report the formation of 'protomorphs'--large globular structures that form in clear solutions of cell extracts--was described. Their formation and their incorporation of amino acids were erratic. During this report year some of the causes of variation have been determined. The formation process is sensitive to concentration. A twofold dilution of the usual pressure cell juice is sufficient to prevent formation, but cell juices prepared by grinding or other processes that preserve intact DNA will form protomorphs from much more dilute solutions. Furthermore, the addition of DNase to the solution invariably prevents protomorph formation. DNA is one of the components of protomorphs, and it seems to be an essential one. Phosphate is also essential. When the cells are very thoroughly washed, protomorphs will not form unless PO_4 is added back to 10^{-3} M. The quantity of intact DNA could easily vary, depending on the conditions in the pressure-cell orifice, and the quantity of PO_4 could vary with the conditions of washing. These factors seem to be sufficient to account for the observed variations in formation of protomorphs.

"The incorporation of amino acids by protomorphs was at times convincing. In particular, the incorporation increased with increased concentration of amino acid or upon supplementation with ATP (conditions that should not affect intact cells). Also, the pattern of amino acid incorporation did not resemble that of whole cells.

"Unfortunately, the incorporation by the protomorphs was also highly erratic. One series of tests showed that the capacity of protomorphs to incorporate amino acids depended on the presence of cell-wall fragments. Again it seems possible to ascribe the observed incorporation to whole cells hidden from view by the protomorphs." Richard B. Roberts.

IV.C.7 Soluble RNA

(Reprinted from Carnegie Institution of Washington Year Book 59, pp. 268-271, 1960.)

E. T. Bolton and R. B. Roberts

SOLUBLE RNA

The theory that soluble RNA acts as a carrier molecule in protein synthesis is widely accepted at present. This concept originated from studies of the incorporation of radioactive amino acids into proteins by cell-free systems. Amino acids linked to S-RNA were transferred to protein when the S-RNA was incubated with ribosomes. Accordingly, it was inferred that the same process was an essential step in protein synthesis as it occurs in the intact cell.

Unfortunately, even the best cell-free preparations are capable of only a very low rate of protein synthesis, usually about 1/1000 the rate observed in cells. Hence it is by no means certain that this residual trace of synthetic activity is qualitatively the same as the process that occurs in the organized cell. Studies of the role of S-RNA in intact cells must be carried out to verify the indications given by the cell-free systems.

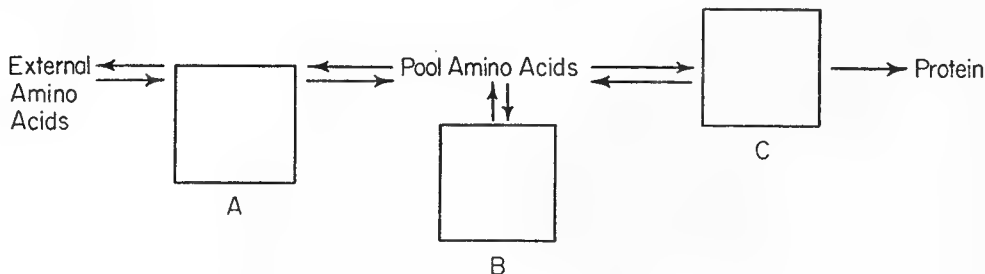
In *E. coli*, $\frac{1}{5}$ to $\frac{1}{6}$ of the RNA is soluble, the rest being associated with protein to form ribosomes. This S-RNA can be distinguished by its sedimentation properties, by its composition, and by its chromatographic behavior on DEAE-cellulose columns. It occurs in units of approximately 100 nucleotides, and each unit is capable of complexing with one molecule of an amino acid.

In relation to the flow of amino acids required to supply the protein synthesis in a growing cell, the S-RNA can provide only a very small pool. The nucleotide content of S-RNA is roughly 100 micromoles per gram dry weight of *E. coli*; thus the maximum quantity of S-RNA-amino acids would be 1 micromole per gram. The rate of increase of protein in a growing culture is 2 per cent per 100 seconds, and the amino acid content of the cellular protein is 5000 micromoles per gram. Hence, the amino acid flow into protein is 1 micromole per second. Accordingly, the S-RNA-amino acid fraction could not supply the requirements of protein synthesis for more than 1 second.

In addition there exists a pool of free amino acids in the cells which, though variable, frequently contains 20 to 100 micromoles of amino acids.

The problem is then to distinguish among the several possible roles of the S-RNA indicated in the diagram below.

In this diagram *A* represents a mechanism for entry into the cell; *B*, a pool in equilibrium with the free amino acid pool; and *C*, a compulsory intermediate of protein synthesis. Alternative *C* is the commonly accepted role of S-RNA. When a radioactive amino acid is added to the medium (or diluted out) its incorporation into the S-RNA fraction may differ, depending on the position occupied by



S-RNA. For example, if S-RNA acts as an entry mechanism (*A*) it will reach its highest specific radioactivity before the free amino acid pool does. On the other hand, if S-RNA acts as precursor to protein (*C*), the rate of incorporation of radioactive amino acids into protein must at all times be proportional to the specific radioactivity of the S-RNA.

Experiments were carried out to check which of these relationships applied to growing cells by using $S^{35}O_4^{2-}$ as a tracer. This particular tracer is most appropriate because pools of cystine and methionine can be depleted by growing the cells for a short period in a sulfur-free medium. During this period the cells utilized their glutathione as a source of sulfur. $S^{35}O_4^{2-}$ was then added to the medium, and samples were taken to determine the incorporation into protein and the S-RNA fraction. At a later time a mixture of nonradioactive cystine, methionine, and SO_4^{2-} was added to dilute out the radioactive

material. Figure 53 shows the time course of incorporation of S^{35} into the S-RNA and protein fractions. The radioactivity of the S-RNA fraction continued to increase after the time when the full rate of incorporation into protein was established. Furthermore, radioactivity persisted in the S-RNA fraction after incorporation into protein had ceased. Thus, the S^{35} bound to the S-RNA cannot be in a form that is an intermediate precursor to protein. Instead it seems to equilibrate with the pool as indicated in *B* of the diagram on page 503.

Because this finding is contrary to current ideas of the role of S-RNA a considerable effort was made to be sure that the S^{35} of the RNA fraction was a true indicator of S^{35} -amino acids linked to S-RNA. This fraction was obtained after three extractions with cold TCA and one extraction with 80 per cent alcohol. The remaining pellet was then suspended in phenol and shaken with buffer, and the

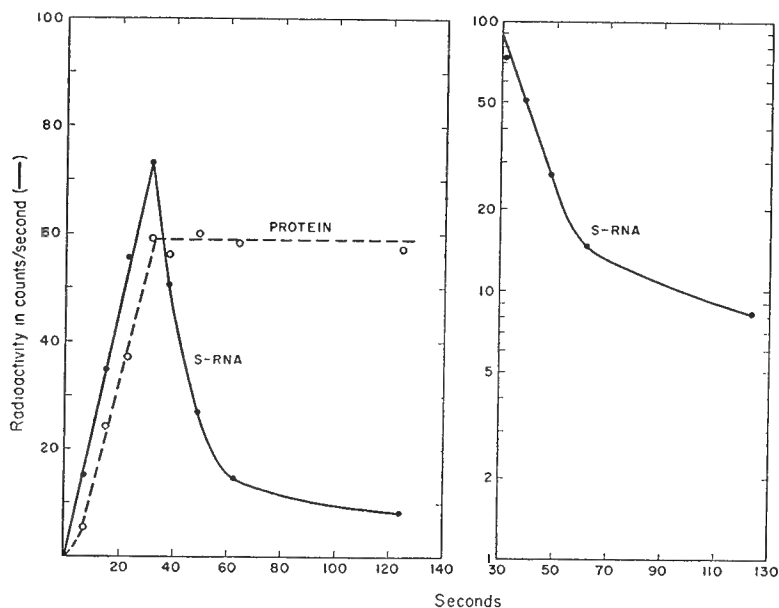


Fig. 53. Cells were incubated ($35^{\circ}C$) with $S^{35}O_4^{2-}$ after 20 minutes' growth in sulfur-free medium. Samples were taken at the indicated intervals and poured into an equal volume of cold 10 per cent TCA. S^{32} -cystine, S^{32} -methionine, and $S^{32}O_4^{2-}$ were added to the culture at the same time sample 4 was poured into the TCA. The incorporation into protein was measured by filtering small samples after extraction with hot 5 per cent TCA. The RNA fraction was purified as described in the text. The maximum quantity of S^{35} found in the S-RNA fraction corresponded to 2 seconds' supply to the protein fraction.

aqueous phase was removed with a syringe. The phenol extraction was repeated three times. Tests with uniformly labeled cells showed that less than 1 part per 1000 of the protein remained. Approximately equal quantities of sulfur radioactivity were obtained by extraction with hot TCA or *M* NaCl. Chromatography of the alcohol-precipitated nucleic acid on DEAE-cellulose showed S^{35} in the same elution pattern as the nucleic acid.

Paper chromatography of the S-RNA fraction showed one unexpected characteristic. The S^{35} remained with the nucleic acid and did not move in a butanol/formic acid/water solvent unless the S-RNA was previously treated with NH_4OH . After this treatment only a portion of the S^{35} showed the movement characteristic of methionine. The major part migrated to the front. Acid hydrolysis of the front material then yielded methionine. The use of NH_4OH to break the linkage of methionine to RNA could yield an amide of methionine, but this would not account for the chromatographic behavior. Rather it appears that there exists a second (alkali-stable) linkage of methionine to a nonpolar material (perhaps lipide). The quantity of this material available was too small for identification. In any event, the proportions of S^{35} in the front component and in free methionine remained constant throughout the time course of incorporation; thus there is no indication of two separate components having different kinetics.

In another experiment the incorporation of S^{35} was stopped by chilling the cells. They were then broken and fractionated by centrifugation before the chemical fractionation. As is shown in table 19, the non-sedimenting RNA carried the bulk of the S^{35} and had by far the highest specific radioactivity.

These experiments with $S^{35}O_4^{2-}$ as a tracer indicated that the $S^{35}O_4^{2-}$ was rap-

idly converted to cystine and methionine, and incorporated into protein. In addition, a small portion of the amino acids was found linked to S-RNA, but the S-RNA-amino acid complex did not show the kinetic behavior of a protein precursor. In many respects $S^{35}O_4^{2-}$ is an ideal tracer, but it must pass through a series of reactions before conversion to amino acids, and these reactions might introduce unforeseen complications. Accordingly, we have attempted to use C^{14} -amino acids as tracers.

To date these experiments have been inconclusive. It has not been possible to cause sufficiently rapid changes in the specific radioactivity of the free amino acid pools to discriminate among the alternative roles of the S-RNA-amino acid complex. Furthermore, the yields of S-RNA-amino acid have been as little as $\frac{1}{10}$ of those expected. At present we believe that further evidence is required before the role of S-RNA in the organization of an intact cell can be established. With the one amino acid that could be measured accurately, the S-RNA complex did not behave like a precursor of protein.

TABLE 19. Distribution of S^{35} among *E. coli* RNA's

Fraction	Component	RNA Content	S^{35} of RNA	Specific Radioactivity
1	70-85S ribosomes	64	19	0.3
2	20-50S ribosomes	25	15	0.6
3	S-RNA	10	64	6.4

Cells were chilled after 40 seconds' incubation with $S^{35}O_4^{2-}$, harvested, washed, and broken in the pressure cell. Cell walls and unbroken cells were sedimented (40K 5' P). Fraction 1 was a 40K 30' P of the 40K 5' SN. Fraction 2 was a 40K 180' P of the 40K 30' SN. Fraction 3 was a 40K 180' SN. RNA was purified from each of these fractions, and its relative quantity and radioactivity were measured.

Comment. This work was done in an attempt to confirm the earlier studies of Lacks and Gros (*J. Mol. Biol.*, **1**, 301-320, 1959). Our results are compatible with theirs, but our conclusions differ. Hendler (*J. Biol. Chem.*, **234**, 1466-1473, 1959), using hen oviduct, has found that the turnover of the SRNA-amino acids is slower than would be expected of an intermediate in protein synthesis. Ellis T. Bolton and Richard B. Roberts.

IV.C.8 Studies with the Mutant *E. coli* 15 T⁻A⁻U⁻

(Reprinted from *Carnegie Institution of Washington Year Book* **59**, pp. 271-277, 1960.)

D. B. Cowie, B. J. McCarthy, and R. B. Roberts

STUDIES WITH THE MUTANT *E. coli* 15 T⁻A⁻U⁻

The *E. coli* mutant 15 T⁻A⁻U⁻ has been studied extensively, because it provides special opportunities to observe the interrelationships among DNA, RNA, and protein synthesis. These cells cannot synthesize thymine (T), uracil (U), or arginine (A), which are components of DNA, RNA, and protein, respectively. Accordingly, it is possible to withhold one or another of these nutrients and shut off the synthesis of DNA, RNA, or protein without any direct effect on the other macromolecules.

Cohen found that thymine-requiring cells lose their viability when incubated without thymine. He attributed this "thymineless death" to unbalanced growth. Maaløe and Hannawalt found the same "thymineless death" when the 15 T⁻A⁻U⁻ cells were deprived of all three requirements, so that no growth was possible. They also found that preincubation with thymine alone (+T-A-U) protected the cells from "thymineless death" in subsequent incubation without thymine (-T-A-U).

Lack of DNA synthesis. When a culture growing exponentially in a medium containing T, A, and U is washed and resuspended in a medium lacking thymine (-T+A+U), growth continues without concurrent DNA synthesis. Division is inhibited immediately, and the cells become enlarged. Figure 54, upper, shows a gradual loss of the capacity to synthesize protein as indicated by incorporation of S³⁵. The capacity to form β-galactosidase is lost abruptly after 50 minutes' incubation, at a time when synthesis of protein is still continuing at a moderate rate (fig. 54, lower).

During this experiment 6 larger samples were taken between 10 and 85 minutes after the removal of the thymine; each sample being given 5 minutes' exposure to S³⁵. They were then fractionated and analyzed on DEAE columns. The distribution of S³⁵ among the soluble proteins remained essentially unchanged, but the proportion of S³⁵ correlated with the nucleoprotein peak was drastically reduced in successive samples. During the 85 minutes the ultraviolet absorption of the ribosome pellet doubled, indicating, as did the incorporation of S³⁵, that ribosomes

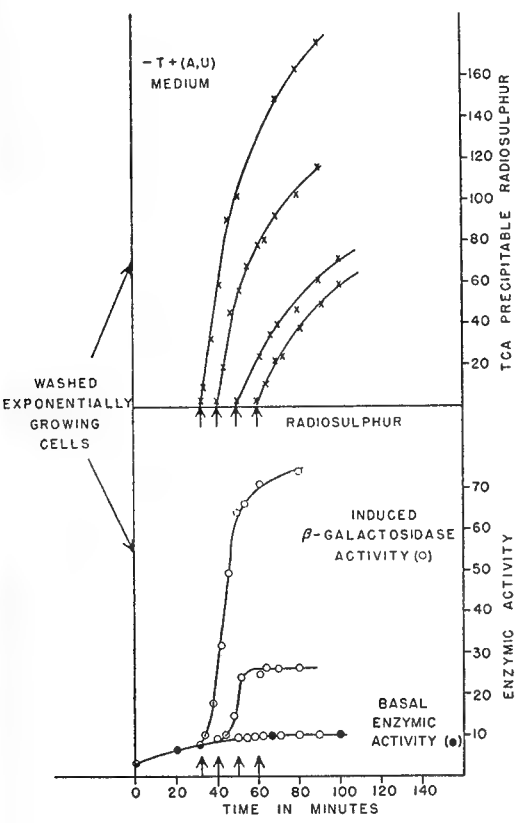


Fig. 54. Sulfur incorporation and enzyme induction during growth without thymine. $S^{35}O_4^{2-}$ and inducer added at times indicated by arrows. Induced enzyme synthesis stops at 50 minutes, but $S^{35}O_4^{2-}$ incorporation continues at reduced rate.

were synthesized although the rate of synthesis decreased. The analytical ultracentrifuge showed a progressive shift in the proportions of the 70 and 85S peaks and the appearance of a new particle group ($\sim 45S$) (fig. 55, pl. 8). The capacity to resume DNA synthesis when thymine was restored after various periods of deprivation showed little change (fig. 56). The capacity to incorporate S^{35} into protein is drastically reduced and the capacity to form β -galactosidase is lost when the cells are incubated for 90 minutes without any of the three nutrients ($-T-A-U$). In contrast, the capacity to incorporate C^{14} -uracil and C^{14} -thymine is much less affected (fig. 57). The ribosome pattern developed no unusual features during this incubation. The capacity to synthesize protein suffers from the simple absence of thymine whether or not arginine and uracil are present. Hence, the effect seems to be due to abnormalities that develop in the DNA rather than to unbalanced growth.

DNA synthesis without RNA or protein synthesis. DNA synthesis without concurrent RNA and protein synthesis can be obtained by incubating the cells with thymine ($+T-A-U$). DNA synthesis

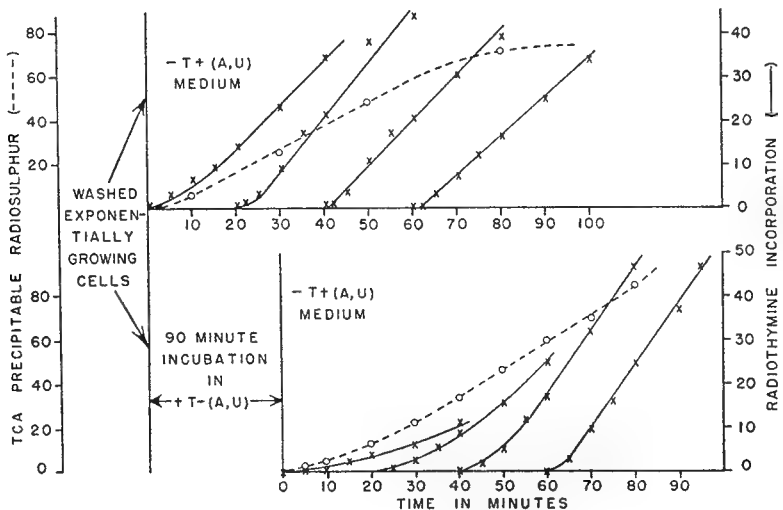


Fig. 56. The capacity to incorporate thymine is little changed after growth without thymine. Cells preincubated $+T-A-U$ are not subject to this reduction.

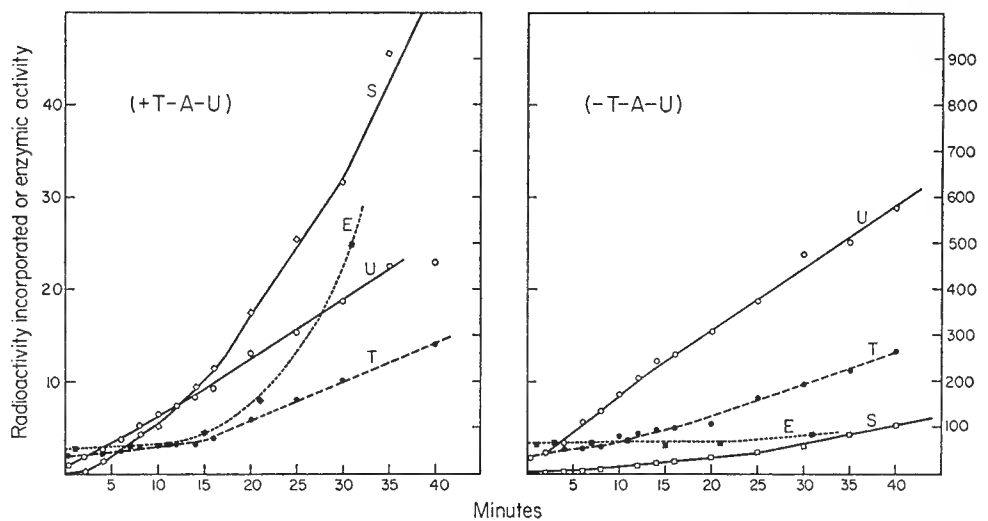


Fig. 57. Cells incubated 90 minutes +T-A-U or -T-A-U, washed, and resuspended in complete medium with inducer. DNA, RNA, and protein synthesis measured by incorporation of C^{14} -thymine (T), C^{14} -uracil (U), or $S^{35}O_4$ (S). β -Galactosidase activity (E). Note loss of capacity to synthesize protein after incubation -T-A+U.

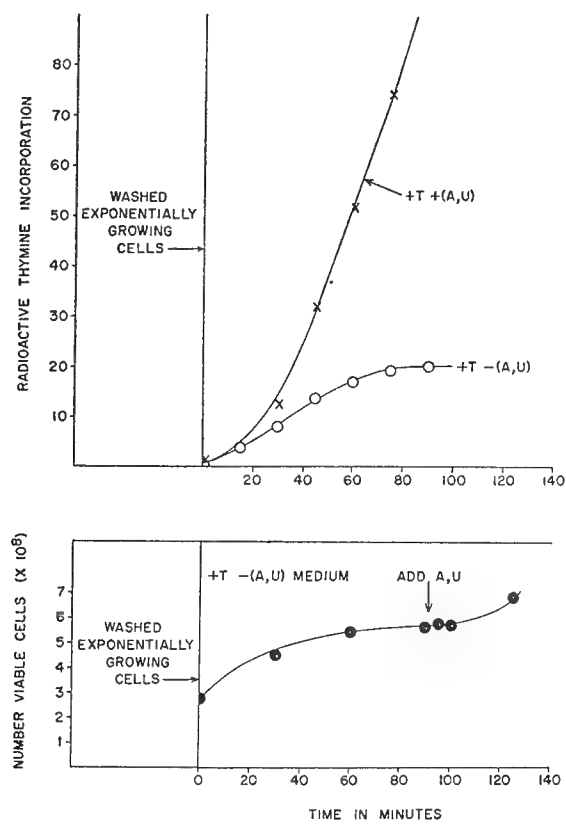


Fig. 58. The DNA content and the number of viable cells increase ~50 per cent during growth +T-A-U.

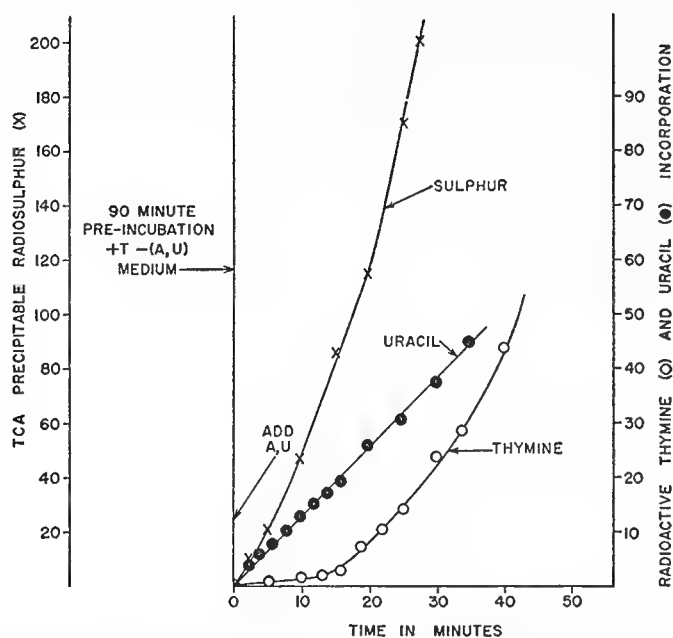


Fig. 59. Thymine-saturated cells show prompt initiation of protein and RNA synthesis when A and U are restored to the medium. The incorporation of thymine shows a delay.

and cell division continue until the number of cells and the quantity of DNA have increased about 50 per cent (fig. 58). After 90 minutes' incubation the cells are quite uniformly small and appear to have only one nucleus. The ribosome pattern shows a high proportion of 85S particles (fig. 61, pl. 9). Cells in this condition are designated "thymine saturated."

Incubation without arginine or without uracil (+T-A+U or +T+A-U) gives much the same result as incubation with thymine alone (+T-A-U). During the 90-minute period there is a slightly greater increase in optical density, but the incorporation of C^{14} -thymine follows the same time course. Incubation without uracil (+T+A-U) causes an almost complete removal of the 70S ribosome peak and a corresponding increase in the 85S peak, leading to a pattern quite similar to that observed in cells incubated without energy source.

Thymine-saturated cells. The properties of these thymine-saturated cells are different from those of an exponentially

growing culture. Maaløe and Hannawalt found that they are not subject to loss of viability when incubated in unsupplemented medium (-T-A-U). Our observations are in agreement with this finding. Furthermore, such an incubation does not bring about any loss of synthetic capacities; the cells are capable of resuming the synthesis of protein, RNA, and DNA when the nutrients are restored. These results indicate that the thymine-saturated cells are unusually stable.

We fully agree with the hypothesis of Maaløe and Hannawalt that the duplication of DNA, once initiated, can go to completion without further RNA or protein synthesis, but that synthesis of RNA and protein is required to permit nuclear division and further DNA synthesis. These thymine-saturated cells then may well be quite uniform in their nuclear configuration as the culture has progressed from a randomly phased condition to a definite stopping point. It seems logical to picture this configuration as four-stranded if the original two-stranded DNA molecules

have duplicated but the nuclei have not yet divided.

The cytoplasm of these cells would not be expected to show any such uniformity. In some cells DNA synthesis would continue for some time after the synthesis of cytoplasmic constituents was halted; in others the DNA synthesis would terminate much earlier. Thus, there might be a two-fold variability in the DNA/RNA ratio from one extreme to the other.

Enzyme Induction in Thymine-Saturated Cells

The thymine-saturated cells show another unusual feature. When uracil and arginine are restored, protein synthesis and RNA synthesis begin promptly (fig. 59). In contrast the synthesis of DNA (fig. 59) and the synthesis of an induced enzyme (fig. 60) require several minutes before the full rate is achieved. The capacity to respond to the inducer develops in these cells whether or not the inducer is present. Furthermore, it develops at the same time as the capacity to synthesize DNA. Thus there seems to be a correlation between the state of the nucleus and the ability to respond to an inducer. Since the correlation might be fortuitous we have attempted to find other explanations for the delay in response to the inducer.

The energy and material required for synthesis of the induced enzyme are available in the cell, as other proteins are synthesized at full rate immediately. Chromatography on DEAE-cellulose of the proteins labeled with S^{35} during the first 3 minutes after restoring arginine and uracil (a period when there is no response to the inducer) showed a normal distribution of the label among the various classes of protein separated by the DEAE-cellulose. The incorporation of S^{35} into TCA-precipitable material therefore measures synthesis of a wide range of proteins and not an abnormal synthesis of a few proteins or polypeptides.

A reduced rate of entry of the inducer cannot be the cause of the low initial rate.

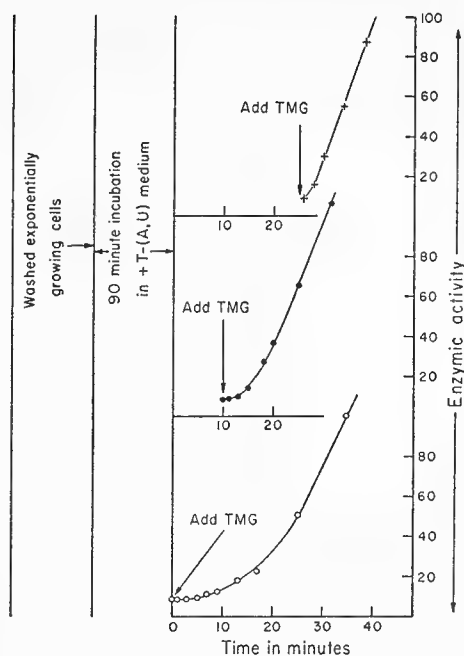


Fig. 60. Thymine-saturated cells show a delay in enzyme induction. The capacity to respond to the inducer develops equally when the inducer is not present.

No change in the rate was caused by adding the inducer 20 minutes before the arginine and uracil or by adding inducer at 100 times the usual concentration. (This concentration, $10^{-2} M$ of IPTG, is sufficient to induce the cryptic mutant.)

There seems to be no reason to attribute the delay to abnormalities in the ribosomes or in the synthesis of ribosomes. During the period of thymine accumulation there is a change in the distribution of ribosomes. The pattern then reverts to the normal pattern of a growing cell during the first few minutes after arginine and uracil are restored (fig. 61, pl. 9). However, energy starvation produces a more extreme alteration in the ribosome pattern, but there it causes no delay in the induction of enzyme. Furthermore, the ribosome pattern reverts to normal in 20 minutes when 5-fluorouracil is added in place of uracil (fig. 62, pl. 10). The usual delay in enzyme induction and in thymine incorporation is then observed when uracil

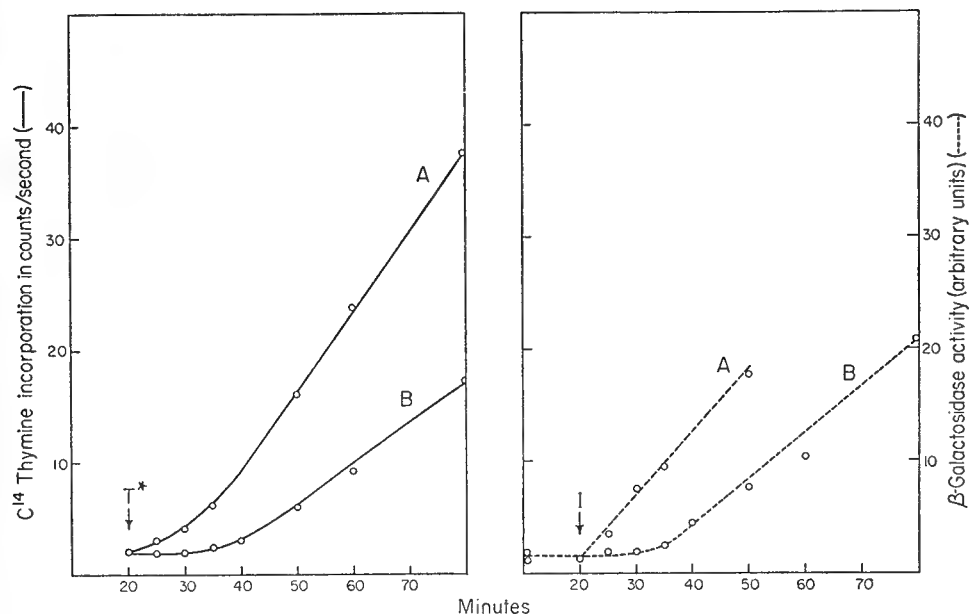


Fig. 63. Cells incubated 90 minutes $+T-A-U$, washed, and resuspended. Culture *A*: incubated 20 minutes $-T+A+U$; inducer, C^{14} -thymine and FU added at 20 minutes. Culture *B*: incubated 20 minutes $-T+A-U+FU$; inducer, C^{14} -thymine and U added at 20 minutes. Culture *A* shows little lag in DNA or enzyme synthesis whereas culture *B* shows usual lag.

is added to reverse the effects of the 5-fluorouracil (fig. 63). Evidently, the material synthesized in the presence of 5-fluorouracil is capable of restoring the ribosome pattern but is not adequate to allow prompt synthesis of DNA or induced enzymes.

The distribution of C^{14} -uracil among the various classes of ribosomes also appeared quite normal when the incorporation occurred during the first 7 minutes after arginine and uracil were restored.

In addition, the ribosomes needed for the synthesis of β -galactosidase appear to be present and active even at the early period, as there is no lag in the synthesis of enzyme at the *uninduced* rate. DNA synthesis is not required for enzyme induction (or other synthesis), as the same initial rate of induced enzyme synthesis occurs whether or not thymine is present.

The delay in enzyme induction might be attributed to the accumulation of a repressor. This view is difficult to prove or disprove by any experiment, but it seems highly unlikely that a repressor of one particular enzyme would cause the same delay in DNA synthesis.

The most obvious conclusion is that the induction of enzyme synthesis can occur only when the cells are in a condition that also allows DNA synthesis. Thus the process of induction may be the result of an association of ribosomes with the nucleus. The exact nature of this association cannot be visualized at present, but it might be one permitting the ribosome to assume a configuration more favorable for protein synthesis.

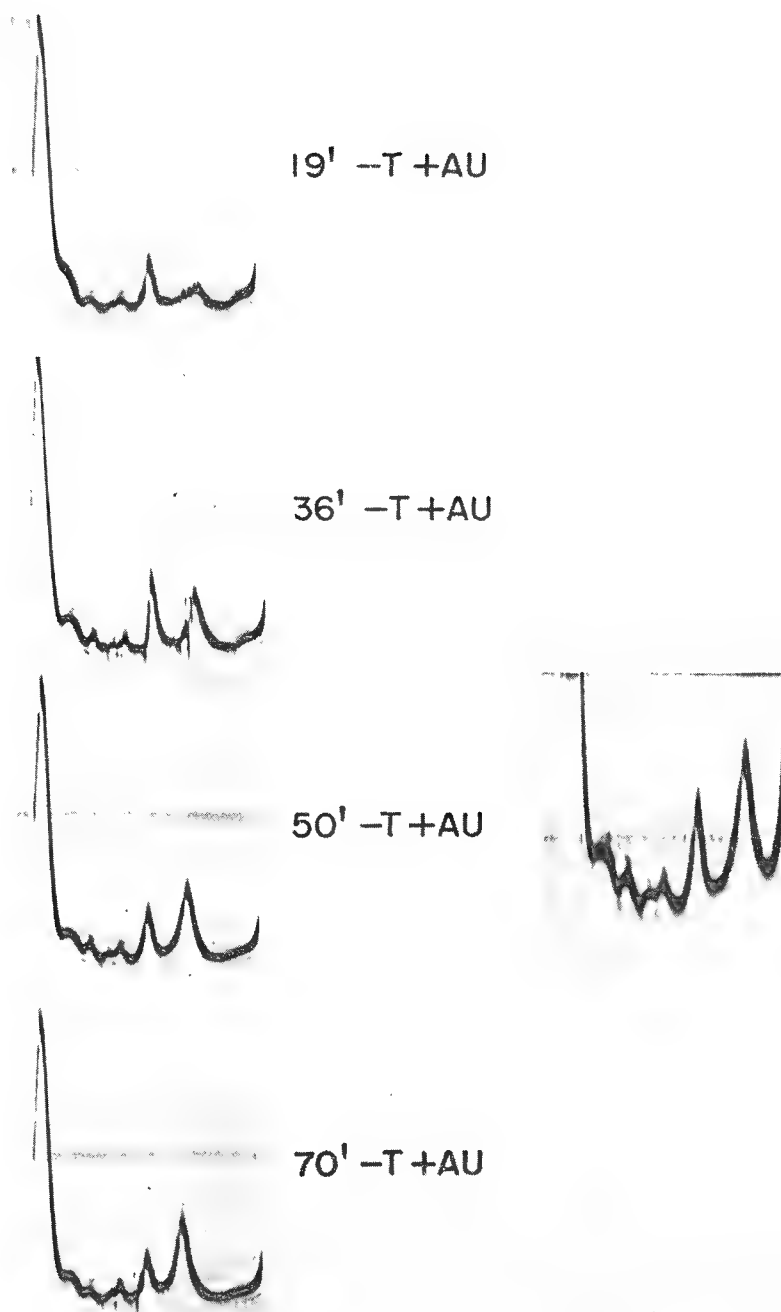


Fig. 55. The analytical centrifuge shows changes in ribosome pattern during growth without thymine. A new peak ($\sim 45S$) appears at 50 minutes. The accumulation of 85S particles is characteristic of a reduced synthetic rate.

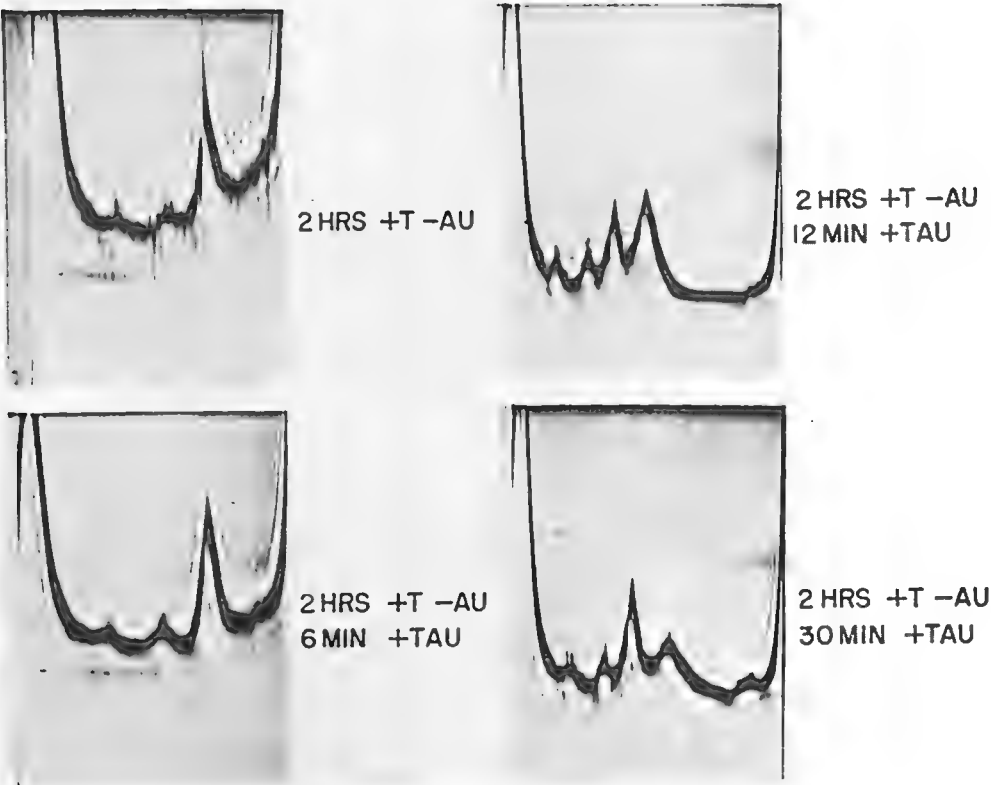


Fig. 61. The ribosome pattern of thymine-saturated cells is characteristic of a low synthetic rate. It reverts rapidly to that of a growing cell when A and U are restored.

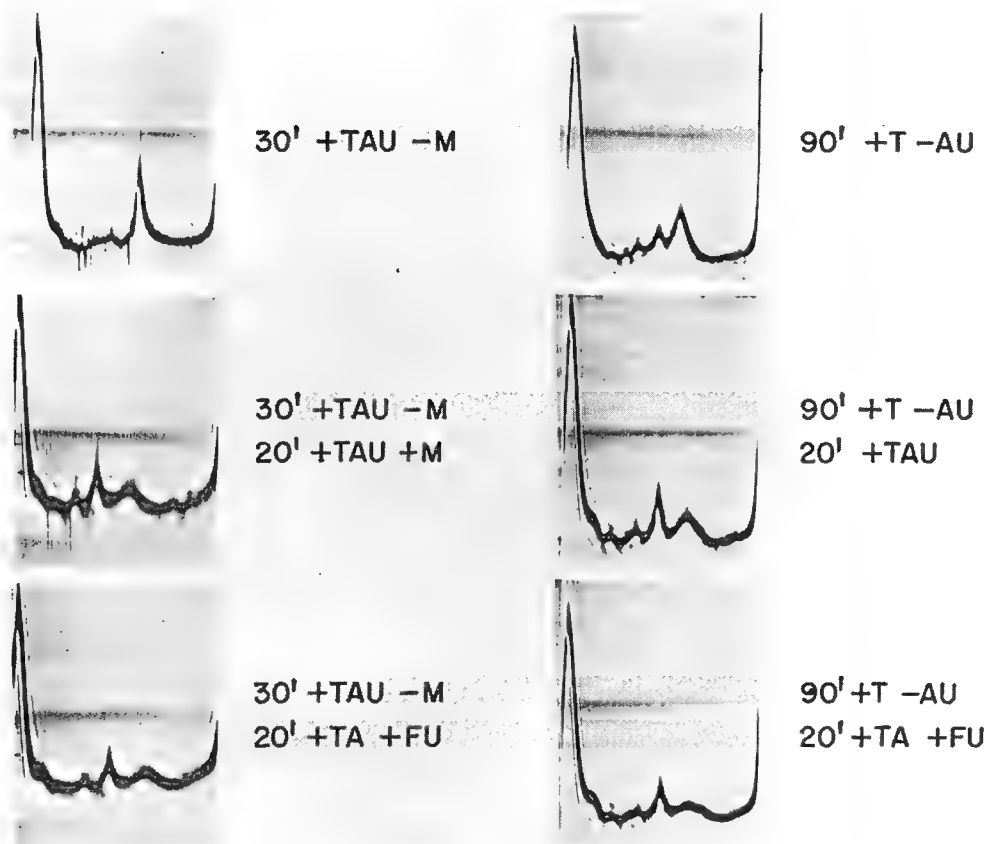


Fig. 62. Thirty minutes' incubation without energy source (maltose) causes accumulation of 85S ribosomes. The pattern reverts to normal in less than 20 minutes when maltose is restored even though uracil is replaced by 5-fluorouracil (FU). Incubation for 90 minutes +T-A-U causes a less drastic change in the ribosome pattern, which also reverts to normal in 20 minutes +T+A+U or +T+A+FU.

Comment. In addition to this report, some of our findings with the mutant *E. coli* 15 T⁻A⁻U⁻ were reported by Roberts in "Synthetic aspects of ribosomes," *Annals of the New York Academy of Sciences*, **88**, 752-769, 1960, and by Cowie, McCarthy, and Roberts at the fourth annual meeting of the Biophysical Society: *Abstracts Biophysical Society*, 1960.

The above results augment and confirm the work of Maaløe and Hanawalt in their investigations of thymine deficiency and DNA replication (O. Maaløe and P. Hanawalt, *J. Mol. Biol.*, **3**, 144-155, 1961; P. Hanawalt, O. Maaløe, D. J. Cummings, and M. Schaechter, *J. Mol. Biol.*, **3**, 156-165, 1961). The lag in β -galactosidase induction observed after thymine saturation is one of the most direct indications that the state of the DNA plays an important role in controlling enzyme expression. Dean B. Cowie.

IV.C.9 Control Mechanisms, Effects of Virus Infection

(Reprinted from Carnegie Institution of Washington Year Book 61, pp. 279-282, 1962.)

D. B. Cowie

Control Mechanisms

In the bacterial cell, protein synthesis and RNA synthesis are closely interlocked. DNA synthesis, in contrast, is relatively independent. Thus, in the 15 T-A-U- mutant the lack of thymine (T) prevents DNA synthesis without any immediate effect on protein or RNA synthesis. Conversely, the lack of arginine (A) and uracil (U) causes no immediate change in the rate of DNA synthesis. The lack of either U or A brings both protein and RNA synthesis to a halt. The requirement of RNA synthesis for the continued formation of protein is a natural consequence of the short lifetime of protein-forming templates. However, there is no obvious reason why amino acids are needed for RNA synthesis.

Protein synthesis is not essential for RNA synthesis, as RNA continues to be synthesized in the presence of concentrations of chloramphenicol that block the incorporation of amino acids into protein. Such results have been interpreted as showing a "catalytic role" of amino acids in RNA synthesis or a "derepression" of RNA synthesis either by amino acids or by their activated forms.

These interpretations do not seem entirely satisfying. The action of chloramphenicol is extremely rapid and much faster than would be expected on this basis. Also, chloramphenicol can remove the inhibition of RNA synthesis caused by an amino acid analog, 5-methyltryptophan. Puromycin allows continued RNA synthesis in the absence of protein synthesis, but it is not as effective as chloramphenicol in eliminating the need for amino acids. Such investigations are being continued in the hope of gaining a

better understanding of cellular control mechanisms.

Effects of virus infection. Last year it was shown that a study of the time course of enzyme synthesis after the addition or removal of an inducer provided valuable clues to the induction mechanism and to regulatory processes in the synthesis of protein. The addition of inducer immediately accelerated the synthesis of β -galactosidase, and after 2.5 to 3.0 minutes a steady rate many times greater than that of the uninduced cells was observed. It was concluded that the brief period of accelerating synthesis was the time required to produce the new enzyme-forming units (EFU) necessary for the induced rate of synthesis of enzyme. Similarly, the removal of inducer caused an immediate deceleration in the rate of synthesis, and within 3 minutes the system returned to the rate observed for uninduced cells. Such a result would be expected if the induced enzyme-forming units were unstable and decayed with a time constant of 2.5 to 3 minutes.

This year other means of markedly altering the rate of protein synthesis were investigated. In collaboration with Dr. Maury Miranda at the Instituto de Biofísica, Universidade do Brazil, Rio de Janeiro, Brazil, studies were carried out to determine the capacity of *E. coli* cells to synthesize host protein after phage infection.

Wild-type T4 bacteriophage rapidly lyses K12 λ *E. coli*, but rII mutants of it are unable to cause lysis of this organism even though infection occurs. Furthermore, Benzer has shown that the rII region of the T4 DNA can be divided into the A and B cistrons, the two subunits having different mutational and func-

tional characteristics. Two rII mutant phages were selected for our studies: 164, a mutant of the A region; and 196, a mutation of the B cistron. K12 λ mixedly infected with both these rII mutants causes efficient lysis of this host and the rapid production of mature phage. Single infection with either the A or B rII mutants does not result in lysis, although the bacteria are no longer capable of cellular replication. Phage development within the host cell could not be demonstrated, and shaking infected cells with chloroform liberated no infective particles.

Infection with rII A phage followed at a later time by a second infection with the complementary rII B phage rendered the K12 λ cells capable of subsequent lysis and phage production. After an elapsed time of several hours between infections phage production still resulted. Evidently the initial infection provides the host cells with certain latent characteristics that can be stored and maintained. This system seemed an ideal one for the investigation of the effect of phage infection upon the capacity of the cell to synthesize host and viral proteins.

The data in figure 52 illustrate the effect of rII phage upon the induced synthesis of β -galactosidase. In this experiment an exponentially growing culture of K12 λ cells was induced for β -galactosidase synthesis with 3.5×10^{-4} *M* methyl-thio- β -D-galactoside (TMG). Nine minutes later rII A phage particles (4 phages per cell) were added to the culture. Figure 52 indicates that the β -galactosidase synthesis continues for several minutes after phage infection. Enzyme production then slows down or stops and later resumes. Plate count analysis revealed that 99 per cent of the cells were infected with the rII A phage. Growth of the culture after infection could not be detected.

In figure 53 it is shown that these enzyme kinetics are quite similar to those observed when glucose (10^{-2} *M*) was added to K12 cells after the induced synthesis of enzyme had been initiated.

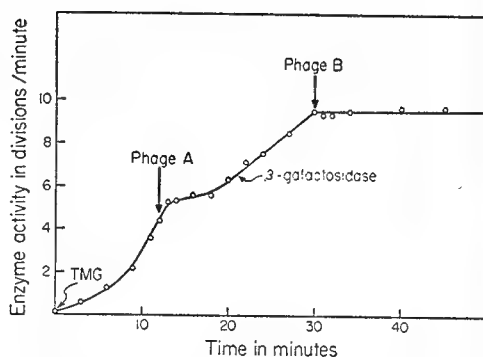


Fig. 52. Kinetics of β -galactosidase synthesis after an initial infection with an rII mutant, A (164 phage), and subsequent infection (36 minutes later) with rII mutant B (196 bacteriophage).

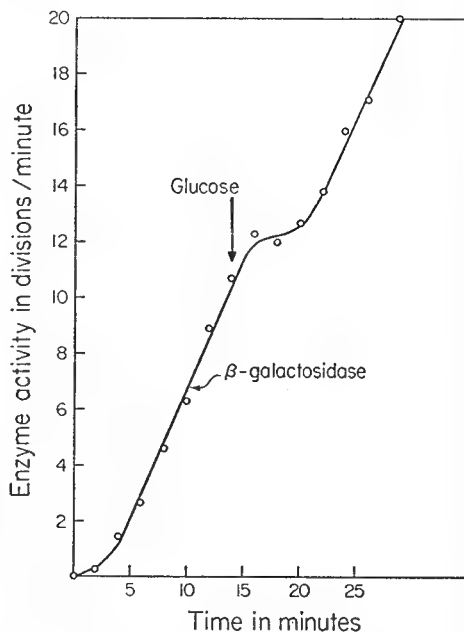


Fig. 53. Effect of glucose on β -galactosidase induction.

The infection with rII A phage (or the addition of glucose) appears to stop the synthesis of active enzyme-forming units. Those already present at the time of infection continue to synthesize enzyme until they decay or become inactive. It is interesting that each of these very different treatments of the culture permits the resumption of the induced synthesis of enzyme. Identical results are

obtained if the culture is infected with rII B mutants instead of the rII A phage.

The temporary inhibition of the synthesis is not caused by low-molecular-weight contaminant material contained in the added phage lysate. Dialysis of the phage suspension does not reduce the effectiveness of the infection. More significantly, nondialyzed preparations added to the culture in which no tryptophan was present caused no effect. Tryptophan is required for the adsorption of these phages to the host.

The synthesis of β -galactosidase can be abruptly stopped after the initial infection with rII A phage if the complementary rII B mutants are added to the culture later. These kinetics are shown in figure 54. Preinduced K12 λ cells were first infected with rII A phage and 18 minutes later with rII B phage. The immediate cessation of synthesis of β -galactosidase is observed as normal phage development begins. Presumably, not only are no new enzyme-forming units synthesized but, more significantly, those

previously existing and functioning after the initial infection are inactivated within seconds.

The rapidity with which phage can stop the synthesis of β -galactosidase can be seen from the data of figure 55. Wild-type T4 phage were added to a preinduced culture of K12 λ . Within seconds all synthesis of β -galactosidase ceased.

It is extremely puzzling that the injection of viral DNA into the host cell should cause instantaneous alterations in the capacity of the cell to synthesize host protein. Ribosomes are presumably the sites for protein synthesis, and it is extremely difficult to imagine a *direct* action of injected DNA on all β -galactosidase-forming units. It is also difficult to imagine why the *complete* phage genome is required for permanent suppression of host protein synthesis.

The information in the injected DNA of one of the rII mutants may remain dormant for long periods but can be expressed at the moment of entry of the second rII mutant DNA. Our current

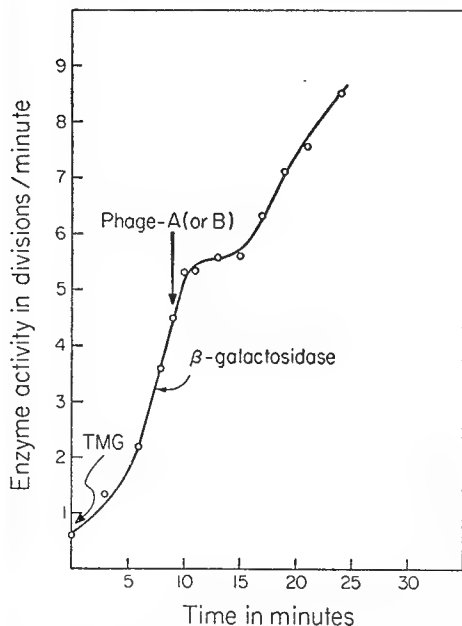


Fig. 54. Time course of induction of β -galactosidase in K12 λ *E. coli* after infection with rII mutants of T4 phage. (A = mutant 164; B = mutant 196.)

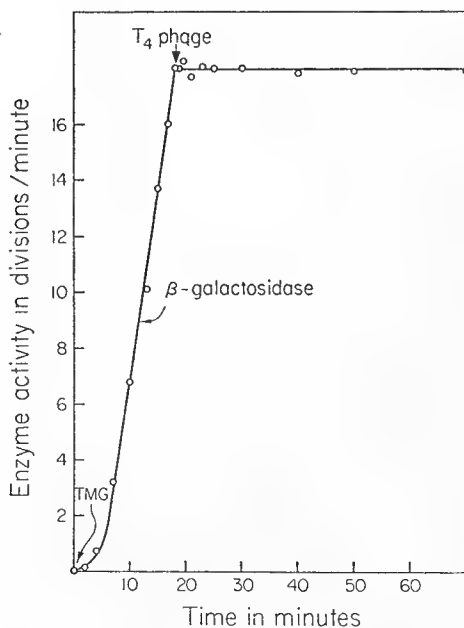


Fig. 55. The kinetics of β -galactosidase induction in K12 λ *E. coli* after infection with wild-type T4 bacteriophage.

research is directed toward determining how and where such information is maintained and the mechanisms by which it is finally expressed.

Comment. The influence of mutant virus (or glucose) in producing temporary cessation of the synthesis of the enzyme-forming units may reflect changes in the cellular organization. Either the state of the DNA (IV.C.8) or the integrity of the polyribosomes may be involved. Dean B. Cowie.

V. SUPPLEMENTARY MATERIAL

A. Virus

V.A.1 Mammalian Viruses and Rickettsiae--Their Purification and Recovery by Cellulose Anion Exchange Columns

(Reprinted, by permission, from Science, vol. 127, no. 3303, pp. 859-863, April 18, 1958.)

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Peterson and Sober (1) and Sober *et al.* (2) described the preparation of the cellulose ion exchangers, DEAE-SF and ECTEOLA-SF, and their use in column chromatography of proteins. The observations of Commoner *et al.* (3) on the chromatography with ECTEOLA-SF of natural tobacco mosaic virus and reconstituted virus nucleoprotein, as well as those of Taussig and Creaser (4) and Creaser and Taussig (5) on chromatography and purification of T1, T2r, and T2r+ bacteriophages with this cellulose ion exchanger, suggested its use in chromatography of animal viruses. This article reports the results of exploratory chromatographic studies on the purification of several representative animal viruses and rickettsiae. It demonstrates that the chromatographic procedure provides high yields of purified infectious agents and discusses some of the implications of the findings for further fundamental and applied research.

Methods

ECTEOLA-SF and DEAE-SF cellulose anion exchangers were used for column chromatography and purification of poliomyelitis (Mahoney, type 1, Lansing, type 2, and Saukett, type 3), ECHO (type 13), and Coxsackie (type A9) viruses from ultracentrifuged concentrates of tissue-culture fluids from infected monkey kidney or Detroit-98 (D-98) cells (6). The behavior of concentrates of Q fever and of epidemic typhus fever rickettsiae from chick-embryo yolk sacs and of Colorado tick fever virus in homogenates of brains from infected suckling mice was also examined.

Preparations of ECHO, Coxsackie, and polio, types 1 and 3, were made from infected monkey kidney cell monolayers in MS solution (7) containing 5 percent calf serum. Polio, type 2, was prepared from D-98 cell monolayers in Eagle's HeLa cell medium (8) with 5

percent horse serum added. Virus concentrates were prepared from tissue-culture fluids clarified by centrifugation at 3500 *g* for 30 to 60 minutes, followed by centrifugation at 100,000 *g* for 1 to 2 hours. The sediments were suspended in 0.01 or 0.02*M* phosphate buffer, *pH* 7.1, then recentrifuged at 100,000 *g* for 1 to 2 hours, and finally resuspended in phosphate buffer. Partially purified concentrates of epidemic typhus and Q fever rickettsiae were made from infected chick-embryo yolk sacs by a modification of Bovarnick and Snyder's method (9). The preparations of Colorado tick fever virus were homogenates of 20 percent (wt./vol.) suspensions, in 0.02*M* phosphate buffer, of brains from infected, moribund, suckling mice.

Several virus preparations were made from appropriately infected tissue cultures grown in the presence of phosphorus-32 in order to label the virus particles with a radioactive tracer. In such cases, MK or D-98 cells were overlaid with Eagle's medium modified to contain 0.001*M* citrate buffer instead of phosphate buffer. To this medium was added 2.5 μ g of KH_2PO_4 per milliliter, 0.01 to 0.07 mc/ml of carrier-free P^{32} as orthophosphate, and dialyzed calf or horse serum (2 or 5 percent).

Columns of cellulose ion exchangers 1 cm in diameter and 7 to 13 cm in length were employed. Such columns contained 1.3 to 2.4 g of ECTEOLA-SF (0.2 milliequivalent/g) or 0.7 to 1.4 g of DEAE-SF (1.0 milliequivalent/g). Preparations of viruses and rickettsiae were loaded onto the columns in 1- to 5-ml quantities and eluted with successive 1-ml quantities of phosphate buffer, or phosphate buffer containing increasing concentrations of NaCl; volumes of 10 to 20 ml of each eluent were used. The usual elution schedule was as follows: 0.01*M* phosphate buffer, 0.02*M* phosphate buffer, 0.1*M* NaCl in 0.02*M* phosphate buffer, 0.25*M* NaCl in 0.02*M* phosphate buffer, and 0.5*M* NaCl in 0.02*M* phosphate buffer. Columns were operated under air

pressure of about 6 lb/in.² with flow rates of approximately 0.5 ml per minute.

Titration of eluates were made with tenfold serial dilutions placed in tubes containing monkey kidney tissue cultures, and the endpoints, in terms of tissue-culture doses (TCD_{50}), were calculated by the method of Reed and Muench (10). Colorado tick fever virus was similarly diluted, although it was necessarily titrated in suckling mice. The radioactivity of dried samples in planchets was assayed with a mica, end-window Geiger-Mueller tube (1.8 mg/cm²) and a decimal scaler.

Rickettsiae

Figure 1 strikingly illustrates that adsorption from 0.02*M* phosphate onto ECTEOLA-SF and elution with 0.1*M* NaCl in 0.02*M* phosphate buffer and 0.2*M* sucrose removed nearly all contaminating material from partially purified suspensions of Q fever rickettsiae. Preparations suspended in 0.02*M* phosphate buffer containing 0.1*M* NaCl and 0.2*M* sucrose were air-dried on Formvar membranes, fixed with osmic acid vapor, washed with distilled water, and shadowed with a platinum-palladium alloy. Yolk-sac material, still present despite differential centrifugation and celite treatment (Fig. 1, top), was removed by one passage through an ECTEOLA-SF column (Fig. 1, middle and bottom). Similar results were obtained with epidemic typhus rickettsiae.

Viruses

Table 1 summarizes the chromatographic behavior of the viruses studied. The viruses, as determined by titration, emerged from the columns in well-defined regions of the eluate and invariably in excellent yield.

ECHO. Preparations of ECHO-13 virus, concentrated from tissue-culture

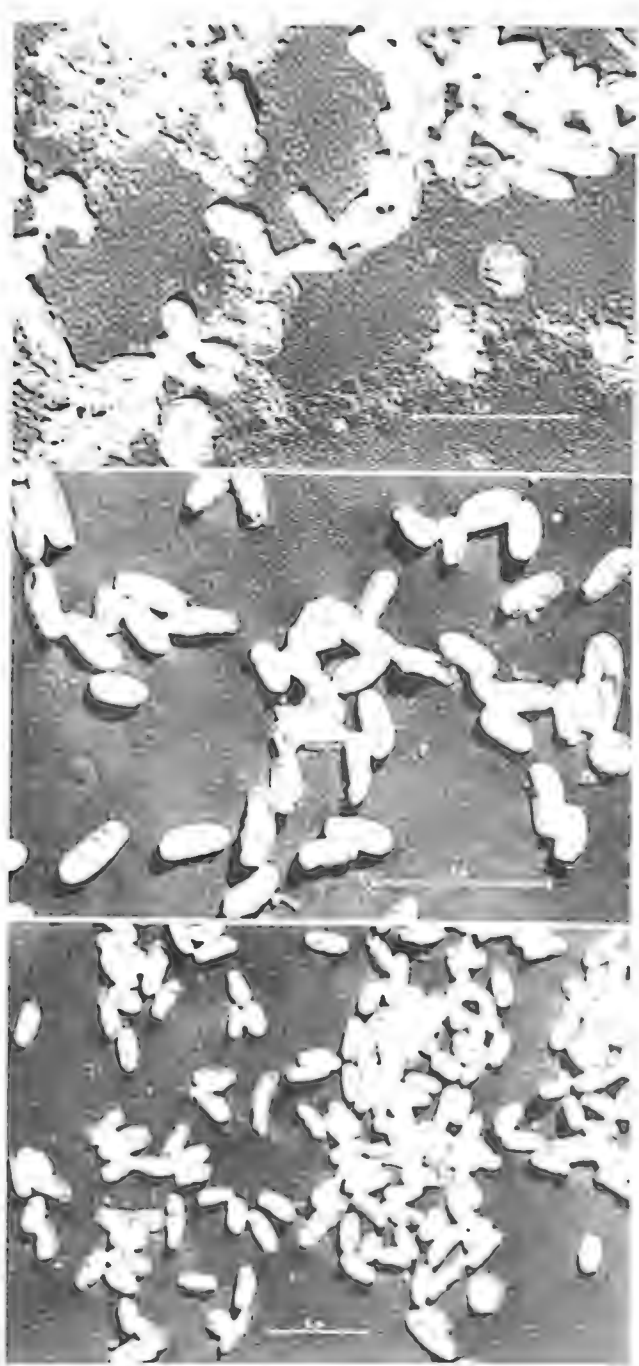


Fig. 1. Electron micrographs of Q fever rickettsiae. (Top) "Purified" (9) rickettsiae. (Middle) Rickettsiae from the same preparation after adsorption onto an ECTEOLA-SF column and elution by 0.1M NaCl at comparable magnification. (Bottom) Typical low-magnification field of a purified preparation. Rickettsiae were prepared for observation and electron micrographs were made by Edgar Ribí and William R. Brown of the Rócky Mountain Laboratory.

fluids from monkey kidney cells infected in the presence of P^{32} , were adsorbed onto ECTEOLA-SF and eluted. Eluates containing viable virus were pooled, concentrated by centrifugation, and read-sorbed onto an ECTEOLA-SF column from 0.01M phosphate buffer at pH 7.1. The virus could again be eluted with 0.1M NaCl (Fig. 2). Results which relate the amount of virus recovered and the radioactivity eluted are illustrated in Fig. 2. This figure shows that 88 percent of the virus recovered was in 1 ml of eluate. This fraction contained most of the radioactivity (0.3 count/min per 2000 tissue-culture doses). ECHO-13 could also be eluted from DEAE-SF by 0.1M NaCl, as determined by titration, although the elution diagram showed appreciable tailing.

Coxsackie. Coxsackie A9 virus, from a P^{32} -labeled tissue-culture fluid concentrate, suspended in 0.02M phosphate buffer, readily passed through an ECTEOLA-SF column, and 98 percent of the added virus appeared in the first few milliliters of effluent (Fig. 3). This effluent contained only 5 percent of the radioactivity initially present in the preparation.

Poliovirus. Polio, type 2, derived from infected, P^{32} -labeled D-98 cells and suspended in 0.02M phosphate buffer was passed through an ECTEOLA-SF column. Titratable virus and radioactivity showed definite correspondence (Fig. 4) although less than 4 percent of the radioactivity added to the column appeared in effluents containing 99 percent of the detectable virus. Most of the titratable virus appeared before the fifth milliliter of 0.02M phosphate buffer had passed through the column (Fig. 4A). The elution of this virus from DEAE-SF was similar to that from ECTEOLA-SF, but less sharp. Effluents which contained virus were pooled and reexamined chromatographically as follows: Without further treatment, one portion was again chromatographed. The elution pattern

was identical with that of the virus-containing portions of the original tissue-culture fluid concentrate (Fig. 4B). About 0.4 counts/min per 2000 tissue-culture-doses were found in the 1-ml fraction which contained most of the detectable virus. Another portion was dialyzed, first against running tap water and then against 0.02M phosphate buffer. Eighty-five percent of the radioactivity was retained within the dialysis bag despite prolonged and repeated dialysis. In addition, 78 percent of the radioactivity of another aliquot was found in the sediment after centrifugation at 80,000 *g* for 100 minutes. The elution diagram of a portion of this non-dialyzable material was identical with that of polio 2 virus derived from the crude concentrate or from column eluates. These findings demonstrated that the chromatographic behavior of the virus remained constant during these treatments.

Ultraviolet absorption spectra of polio 2 preparations (10^{10} TCD₅₀/ml) purified by passage through an ECTEOLA-SF column possessed a characteristic nucleoprotein absorption peak at 260 mμ which was not apparent in the spectrum of the input material (Fig. 5).

Poliovirus, type 1, concentrated by centrifugation, washed, and resuspended in 0.01M phosphate buffer, pH 7.1, was added to an ECTEOLA-SF column. The major portion was recovered in the first eleven milliliters of 0.01M phosphate buffer eluent. Type 3 poliovirus in tissue-culture fluid, diluted 1:10 with 0.02M phosphate buffer, pH 7.1, and added to an ECTEOLA-SF column, was recovered in the first five milliliters of the initial eluent, 0.02M phosphate buffer. These findings do not necessarily imply different elution characteristics for these polioviruses, since the initial conditions were not comparable.

Colorado tick fever. Five milliliters of Colorado tick fever virus, in the form of 20 percent mouse-brain homogenate,

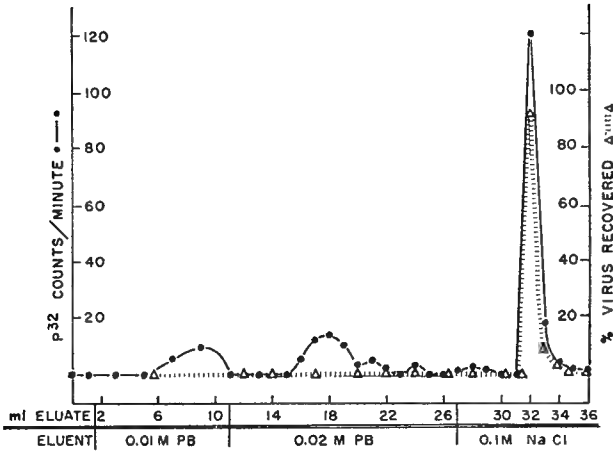


Fig. 2. Relationship between virus recovery and radioactivity (P^{32}) from a preparation of ECHO-13 virus grown on P^{32} -labeled monkey kidney cells, adsorbed onto an ECTEOLA-SF column, and eluted by 0.1M NaCl.

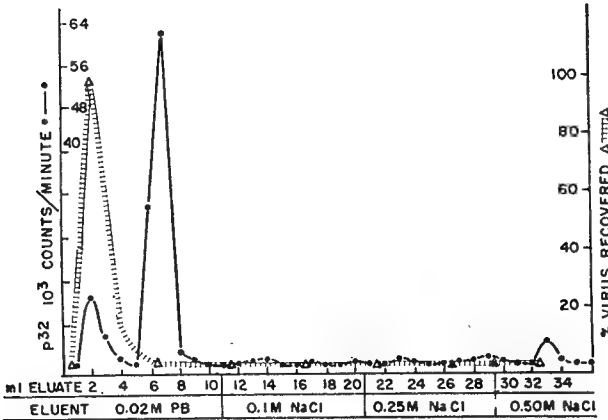


Fig. 3. Relationship between virus recovery and radioactivity (P^{32}) from a preparation of Coxsackie A9 virus grown on P^{32} -labeled monkey kidney cells, adsorbed onto an ECTEOLA-SF column, and eluted by 0.02M phosphate buffer.

Table 1. Summary of elution characteristics of viruses and their recovery from ECTEOLA-SF and DEAE-SF columns.

Agent	Source	Eluent*	Titers of virus†	
			Total added	Eluate
Polio 2	Tissue-culture fluid	0.02M phosphate buffer	9.0	8.8
Polio 2	Tissue-culture fluid	0.02M phosphate buffer*	8.6	8.6
Colorado tick fever	Mouse brain	0.5M NaCl	8.9	8.8
ECHO-13	Tissue-culture fluid	0.1M NaCl	5.3	5.4
ECHO-13	Tissue-culture fluid	0.1M NaCl*	6.8	6.8
Coxsackie A9	Tissue-culture fluid	0.02M phosphate buffer	9.6	9.3
Polio 1	Tissue-culture fluid	0.01M phosphate buffer	7.8	8.0
Polio 3	Tissue-culture fluid	0.02M phosphate buffer	7.5	7.5

* Virus first appeared in large quantity when columns were eluted with phosphate buffer or PB + NaCl in the concentration noted. Those eluents marked by asterisks indicate that results were obtained with DEAE-SF; all others were obtained with ECTEOLA-SF.

† Virus titrations are expressed as log₁₀ of the TCD₅₀ or LD₅₀. The titer in the column "Eluate" refers only to the virus released by the eluent noted in column 3.

were adsorbed onto a 10-cm ECTEOLA-SF column. Elution results are given in Table 2. Most of the titratable virus (67 percent) was eluted by 0.50M NaCl in phosphate buffer, whereas only 13 percent of the total protein added to the

column was recovered in this fraction. Thus, partial virus purification was achieved, even from very crude starting material, and virus recovery was satisfactory.

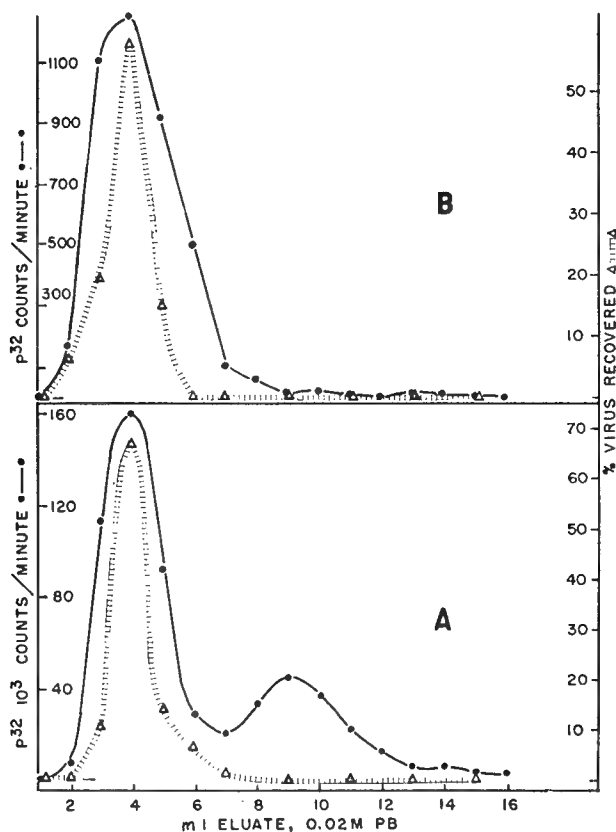


Fig. 4. Relationship between virus recovery and radioactivity (P^{32}) from a preparation of polio 2 virus grown on P^{32} -labeled Detroit-98 cells, adsorbed onto an ECTEOLA-SF column, and eluted by 0.02M phosphate buffer. Elution diagrams in A were obtained from 2.3 ml of crude concentrate. The third to sixth milliliters of eluates from A were pooled and diluted; 2.3 ml of this pool was adsorbed onto and eluted from a duplicate column of ECTEOLA-SF to give the elution diagrams presented in B.

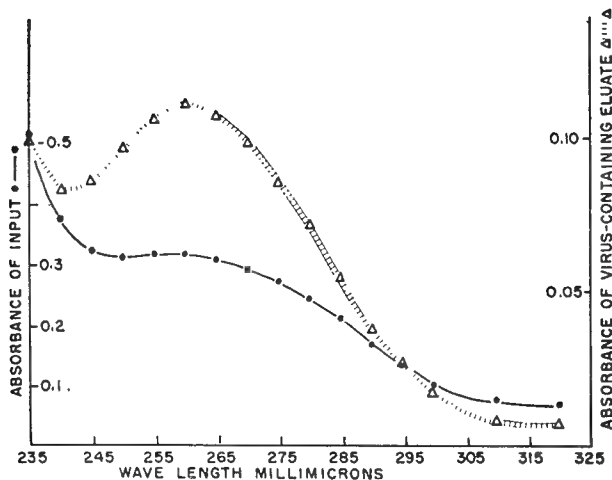


Fig. 5. Absorption spectrum of a crude polio 2 virus preparation compared with the spectrum of the virus-containing eluate obtained after the crude preparation had been adsorbed onto an ECTEOLA-SF column and eluted by 0.02M phosphate buffer.

Table 2. Behavior on an ECTEOLA-SF column of Colorado tick fever virus from a mouse-brain homogenate.

Preparation	Number of LD ₅₀	Percentage of recovered virus	Protein content of eluates
Original	8×10^8		86 units*
0.02M eluate	0	0	8.8 units (10)†
0.10M eluate	1×10^8	11	9.2 units (11)
0.25M eluate	2×10^8	22	13.1 units (15)
0.50M eluate	6×10^8	67	11.1 unit (13)
Total recovered	9×10^8		

* A unit is equivalent to 270 μ g of standard crystallized bovine albumin (Armour and Co., Chicago, Ill.) as determined by the procedure of Lowry *et al.* (11).

† The figures in parentheses indicate the percentage of original protein recovered in the eluate.

In the experiments described, material loaded onto the columns (except for the Colorado tick fever suspension described above) generally contained from 0.2 to 1.0 mg of protein [0.7 to 3.5 absorbance units determined by the procedure of Lowry *et al.* (11); protein values are expressed in terms of a standard solution of crystallized bovine albumin, Armour and Co., Chicago, Ill.]. Eluates with the major portion of titratable virus usually contained less than 20 μ g of protein per milliliter. When these eluates were again adsorbed and eluted, protein was generally not detectable (less than 1 μ g) in the virus-containing fractions, although most of the virus was recovered.

Discussion

The foregoing data indicate that rickettsiae and some mammalian viruses can be adsorbed onto and selectively eluted from ECTEOLA-SF and DEAE-SF columns. The conditions under which this can be accomplished also permit the recovery of essentially all the virus added to the columns and allow the removal of much extraneous substance.

Coxsackie A9, and poliovirus, types 1, 2, and 3, did not appear to be adsorbed under the conditions employed, since they appeared in the very early fractions of the initial eluents. In spite of this behavior, a considerable degree of purification can be achieved. For example, most of the contaminating, phosphorus-containing material can be

adsorbed by the ion exchanger and can be separated from the infectious particles. Furthermore, most of the extraneous protein can be adsorbed by the ion exchanger and also separated from the virus. The close correspondence between virus activity and P³² distribution also indicates that marked virus purification was accomplished with ECHO-13 and polio 2 viruses. These data indicate the practicality of simply and effectively labeling and purifying useful quantities of infectious mammalian viruses. Such viruses may well provide a much-needed material for studying virus-host relationships and the antigenic properties of animal viruses.

Rickettsiae, as exemplified by the organisms of Q fever and epidemic typhus, can be adsorbed and purified on cellulose exchangers. Rickettsial preparations of the purity indicated by the electron micrographs (see Fig. 1) are of obvious significance for studying rickettsial chemistry, metabolism, and immunology.

It is of particular interest that differences in chromatographic behavior, irrespective of particle size, have been demonstrated. Such different behavior must be presumed to be due to intrinsic differences in the surfaces of these agents. This is strongly suggested by the work of Taussig and Creaser on the coliphages (4, 5) in which the virus behavior in a column was shown to be determined by the character of the protein moiety. Among the viruses examined in the present investigation, ECHO-13 and Colo-

rado tick fever are definitely different from each other as well as from the group comprising poliovirus, types 1, 2, and 3, and Coxsackie A9. ECHO-13 was eluted by 0.1M NaCl, whereas Colorado tick fever was eluted principally by 0.5M NaCl; in contrast, the other species studied passed through the column at very low salt concentrations. The character of the brain suspension may have modified the behavior of the Colorado tick fever virus on the column, but subsequent experiments with preparations containing very small amounts of brain material indicated similar elution behavior.

The experiments reported above are essentially exploratory. Nevertheless, the simplicity of this type of purification and examination of animal viruses encourages the hope that studies of these viruses will be concerned as much with the virus itself as with contaminating host material. Elimination of contaminating host material from virus vaccines may be very important in reducing "side effects," such as allergic encephalitis, from vaccines which can be obtained only from tissues of whole animals. Chemical inactivations of viruses may also be carried out in systems relatively free of contaminants which would normally react with the inactivating agents and, thus, alter their effects on the viruses themselves. Differences in elution characteristics of the viruses presently investigated strongly suggest that surface properties of animal viruses can be investigated by means of chromatographic methods. In this connection, preliminary experiments (12) have shown that formaldehyde-treated polio 2 can be separated from untreated virus.

In general, we feel that procedures such as those described offer the definite hope that studies of animal viruses will no longer be hampered by the past difficulties involved in preparation of purified agents.

Summary

Techniques of column chromatography with cellulose ion exchangers have been successfully applied to mammalian viruses and rickettsiae. Recovery of virus is excellent, and appreciable purification in terms of phosphorus and protein removal has been demonstrated.

Elution characteristics of poliovirus (types 1, 2, and 3), and Coxsackie A9 virus are similar, whereas those of ECHO-13 and Colorado tick fever differ from them as well as from each other.

Elution diagrams of preparations of ECHO-13 and polio 2 viruses grown on P³²-labeled tissue cultures show a high degree of correlation between the distribution of titratable virus and the distribution of radioactivity.

A single adsorption and elution of Q fever or epidemic typhus fever rickettsiae results in a striking degree of purification, as demonstrated by electron micrographs.

The chromatographic behavior of the animal viruses and rickettsiae appears to depend more upon the chemical nature of the surfaces of these infectious agents than upon their size.

The chromatographic procedure described may prove useful in the preparation of purified, P³²-labeled, fully infectious animal viruses for further fundamental research. It may also prove useful for the removal of unwanted host materials in the preparation of vaccines.

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V.A.2 Simple Method for Preparation of Purified Radioactive Poliovirus Particles: Electron Micrograph

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P³²-Labeled poliovirus can be produced in radioactive tissue culture cells and purified by cellulose ion exchangers (1). Highly purified and radioactive poliovirus particles with characteristics similar to those reported by Schwerdt and Schaffer (2, 3) have now been prepared from 100 to 600 ml of media by a simple, rapid procedure involving column treatment and centrifugation. The essentials of this procedure are outlined below.

A. Monolayers of monkey kidney cells were washed with Hanks' salt solution modified to contain 0.001 *M* citrate instead of phosphate. One to ten plaque-forming units (PFU) type 2 (Lansing) poliovirus per cell were adsorbed to the monkey kidney cells for 1 hour at 36° from a small volume of Eagle's (4) HeLa cell medium containing 0.001 *M* citrate, pH 7.0, instead of phosphate, 0.21% NaHCO₃, and 5% horse serum which had been dialyzed against citrate-Hanks. After adsorption, 2 ml modified Eagle's medium, with 0.013 mc carrier-free P³²-orthophosphate per milliliter, was added per 10⁶ monkey kidney cells. Monolayers of uninfected cells removed about 50% of the P³² from this medium after 24 hours at 36°.

B. After 48 hours, the medium and cell mixture was frozen and thawed, the debris removed by centrifugation at 1000 *g* for 20 minutes, and the clarified supernatant (10⁷ to 10⁸ PFU/ml) centrifuged at 80,000 *g* for 90 minutes. The pellet was thoroughly suspended in 4-8 ml 0.02 *M* phosphate buffer, pH 7.1.

C. The pellet from "B" was placed on a 12 × 100-mm DEAE Solka-Floc column (5), capacity about 1 meq/g, adequate to process a suspension derived from 1000 ml medium. Phosphate buffer, 0.02 *M*, pH 7.1, was used as eluent and 3-ml quantities of eluate were collected in individual tubes. The tubes of eluate, corresponding to the first peak of radioactivity, were pooled. This pool contained most of the titratable virus (1), and the ratio of optical densities at 260 and 280 *mμ* was 1.0 to 1.4.

D. The pooled eluates of "C" were centrifuged at 100,000 *g* for 90 minutes. The ratio of optical densities at 260 and 280 *mμ* was 1.6 to 1.7 for pellets suspended in distilled water. At this stage the electron microscope revealed poliovirus embedded in a homogeneous layer of very fine particles (possibly protein), estimated to be 5-10% of the total material present.

E. The suspended pellet from "D" was centrifuged at 10,000 *g* for 20 minutes in a 2-ml tube. The supernatant was carefully removed and the sediment discarded. The ratio of optical densities at 260 and 280 *mμ* was about 1.7 and the specific infectivity 1-10 × 10¹³ plaque-forming units (PFU) per gram protein. The absorption spectra were similar to the spectrum presented by Schwerdt and Schaffer (2). Radioactivity was usually 5 counts per minute per 10⁴ PFU as determined from dried samples by a Nuclear-Chicago gas-flow counter with a "micromil" end window.

In a typical experiment there were $4.4 \pm 1.5 \times 10^{10}$ PFU in the step "B" suspension and $2.2 \pm 0.2 \times 10^{10}$ PFU in the supernatant, step "E". Using the step "C"

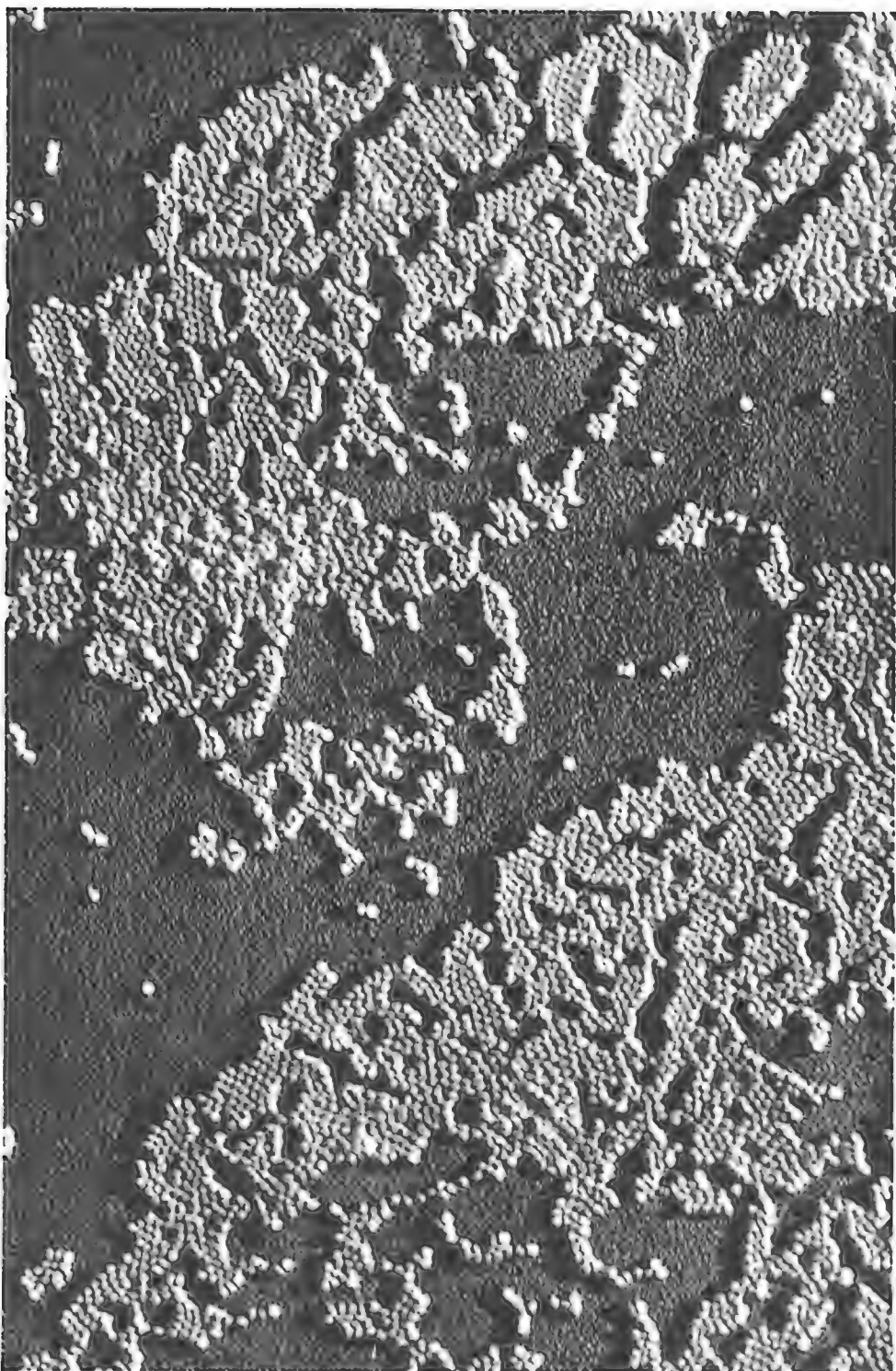


FIG. 1. Purified type 2 (Lansing) poliovirus; particles average 25 $m\mu$ in diameter. Electron micrograph by Edgar Ribí and W. R. Brown. Magnification: $\times 100,000$.

pool as the reference, 62% of the radioactivity present was recovered in the step "E" supernatant.

Electron microscopy was done on pellets obtained by centrifuging aliquots of supernatant from "E" at 100,000 *g* for 90 minutes. The pellets were suspended in small volumes of distilled water and placed on Formvar membranes overlaying highly oxidized copper screens; the aqueous menstruum wetted such a screen, and part of the particles were adsorbed. After a short adsorption period, the excess liquid was removed from the first screen and placed on the second. This process was repeated on a series of screens until the entire pellet was adsorbed. All screens in a series yielded virus as clean as that indicated by the electron micrograph presented (Fig. 1).

Preparations of the purity indicated in Fig. 1 yielded crystals when 2.5 mg/ml suspensions of virus, in distilled water, were incubated overnight at 4°. About 90% of the radioactivity and virus activity was present in the pellet when these crystallized suspensions were centrifuged at 5000 *g* for 10 minutes; the ratio of optical densities at 260 and 280 *mμ* was about 1.7 for pellets (crystals) resuspended in 0.02 *M* phosphate buffer, pH 7.1.

Radioactive virus was not essential for this procedure, but radioactivity and virus activity were proportional in "C", "D", and "E" and yields were easily determined at each step.

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V.A.3 Kinetics of Labelling of Turnip Yellow Mosaic Virus with P^{32} and S^{35}

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The time course of labelling of the various classes of viruslike particles that can be isolated from plants infected with turnip yellow mosaic virus has been followed using S^{35} to label protein and P^{32} to label RNA. A theoretical treatment for the relation between virus protein and nucleoprotein is presented. The labelling data eliminate certain of the theoretically possible relationships, but a number of alternatives remain. The simplest model consistent with the data is one in which the protein is formed reversibly and the nucleoprotein irreversibly from the same pool of precursor protein subunits. The minor nucleoprotein components cannot be stages in the assembly between the empty protein shell and complete virus. Various attempts to carry out pulse type labelling experiments with P^{32} in leaves of *Brassica pekinensis* were unsuccessful.

INTRODUCTION

For several viruses, both plant and animal, noninfectious viruslike components have been isolated from infected tissues. Frequently such components contain less than the full complement of RNA, or none at all. In no instance is the relationship of the incomplete virus to the infectious particle established beyond doubt. In the most common type of experiment on this problem, cells or tissues in which virus is multiplying are given a period of exposure to some suitable radioactively labelled compound. The components are then isolated and the specific activity of the two or more components is determined. In many such experiments periods of hours or even days of exposure to the label have been given [e.g., Delwiche *et al.* (1955) and Commoner and Rodenberg (1955) with tobacco mosaic virus]. Such lengthy periods of labelling would be likely to obscure differences in the rates of incorporation of a labelled precursor.

On the basis of short-term labelling tests with $C^{14}O_2$ (Jeener, 1954) and S^{35} -labelled sulfate (Matthews, 1960), it has been

suggested that the empty virus protein shells found associated with complete virus in turnip yellow mosaic virus (TYMV) infections are precursors of the complete virus. However, as pointed out by these authors, the results by no means demonstrated a precursor-product relationship. In the experiments described in this paper we have explored the possibility of carrying out kinetic labelling tests with TYMV. Although our experiments do not establish the precise relationship between the two types of particle they do allow certain possibilities to be eliminated, and they suggest the kind of experiments that will be necessary to resolve the problem definitively.

MATERIALS AND METHODS

Culture of virus. TYMV (Cambridge type strain: Markham, 1959) was cultured in Chinese cabbage (*Brassica pekinensis*, Rupr. 'Wong Bok') grown in pots in the glasshouse.

Isolation of virus. Where purified preparations of TYMV were required, the pH 4.8

procedure (Matthews, 1960) was used. Infectious nucleoprotein (B_1), empty protein shell (T), and the minor nucleoprotein fractions (B_{000} , B_{00} , B_0 and B_2) were isolated from purified preparations by 2–3 cycles of density gradient sedimentation in dense cesium chloride solutions (Matthews, 1960).

Labelling procedures. We tested a variety of methods for introducing P^{32} labelled orthophosphate and S^{35} -labelled sulfate into Chinese cabbage leaves. These included floating leaf disks, vacuum-impregnated leaf disks, and excised leaves with petioles standing in the solution. However none of these were as satisfactory with respect to speed and efficiency of uptake as the whole plant procedure previously used with TYMV (Matthews, 1960). This method, in which the washed roots of an intact plant are placed in a small volume of labelled solution, and in which radioactivity in the leaf reaches a more or less constant level in about 1 hour, was used routinely in the present work. For measurements of radioactivity, aliquots of liquid samples were dried on 25-mm planchettes and counted with a GM tube (Phillips PW18505).

Analytical procedures. Nitrogen was determined by the micro-Kjeldahl procedure using the apparatus of Markham (1942). Phosphorus was estimated by the method of Allen (1940). Ultraviolet absorption measurements were made in a Beckman DU spectrophotometer using cells of 1-cm path length. Paper chromatographic and electrophoretic procedures were those summarized by Markham (1955).

Serological tests. The serological-chromatographic procedure (Matthews, 1954) was used to isolate and estimate amounts of RNA associated with TYMV protein in samples of heat-clarified leaf sap. This procedure would include RNA from the minor TYMV nucleoprotein components described by Matthews (1960).

Partition of total leaf phosphorus. Freshly cut disk samples of leaf tissue weighing 10–20 mg were boiled for 3 minutes in 3 ml of 70% ethanol made 0.1 *N* with respect to acetic acid. The ethanol was poured off, and a second extraction with 2 ml of 95%

ethanol was carried out. The two ethanol extracts were combined and used to give an estimate of "ethanol-soluble P." The extracted disks were dried at 95° and used for the estimation of insoluble P. Tests on several sets of P^{32} -labelled leaf material showed that this procedure gave partition of P both with respect to quantity and specific activity very similar to the more commonly used extraction with cold 10% trichloroacetic acid.

Estimation of amount of virus per leaf. In many published studies of virus increase in leaves, results are expressed as weight of virus material per unit fresh weight (F.W.) of leaf or per unit volume of leaf sap. These expressions yield an underestimate of virus increase if leaves are expanding in area or thickness with time. In the labelling experiments described here, virus increase was determined on a weight of virus per leaf basis. The most precise estimates of virus increase are obtained if the same set of leaves are sampled throughout an experiment. The following procedure allowed us to estimate total virus in the leaf without removing the leaf from the plant. Disk samples of leaf were taken at each time. These were used to give an estimate of weight of virus per unit fresh weight of leaf and of fresh weight of leaf per unit area. To determine leaf area, leaf width at the widest point was measured at each time of sampling and converted to area by the use of a factor that had been previously determined using a similar batch of plants. Over the range of leaf size used (90–150 cm²) the relation between leaf width and area was nearly linear. The leaf area multiplied by the fresh weight per unit area determined from the disk samples gave an estimate of the fresh weight of the green part of the leaf at each time. (The midrib was avoided in taking disks.)

EXPERIMENTAL

Attempts to Alter Phosphorus Levels in the Chinese Cabbage Plant

To assess the feasibility of carrying out "chase" type experiments with P^{32} -labelled

TABLE 1
DISTRIBUTION OF PHOSPHORUS IN THE CHINESE CABBAGE PLANT

Organ	Concentration of P ($\mu\text{g P/mg fresh weight}$)			Total P in organ (μg)		
	Soluble ^b	Insoluble	Total	Soluble	Insoluble	Total
Small ^a leaf lamina	0.11	0.08	0.19	21	13	34
Large leaf lamina	0.12	0.05	0.17	610	260	860
Large leaf petiole	0.033	0.007	0.041	192	41	240
Tap root	0.092	0.029	0.12	22	6.9	29
Hair roots	0.057	0.035	0.092	41	26	67

^a Small leaf approximately 2 cm in length. Large leaf 16 cm in length.

^b Soluble or insoluble in hot 70% ethanol.

orthophosphate in TYMV-infected Chinese cabbage plants, we determined P levels in these plants under various conditions.

Distribution of phosphorus in the plant. Table 1 shows the distribution of P in various organs of a Chinese cabbage plant grown under the conditions, and at the stage of growth, used for virus experiments. The data suggest that in a plant with three "small" leaves and three "large" leaves, about 80% of the total P in the plant would be in the laminae of the larger leaves. This is almost certainly the main site of synthesis for TYMV. In a fully infected plant, virus concentration is some 20 times higher in the leaf laminae than in other parts of the plant.

Effect of TYMV infection on P levels in leaves. In inoculated leaves and in systemically infected leaves, tested at various times after infection, TYMV caused no consistent alteration in the total amount of P in the leaf or in the partition of the P into ethanol soluble and insoluble fractions.

Attempts to lower P levels in leaves. Two procedures—P starvation of whole plants, and maintenance of leaves removed from the plant—were tested in the hope that P levels could be reduced.

Plants were transferred from soil to washed sand and supplied for 4 weeks with a liquid nutrient medium containing no P. By this time they were severely stunted and showed purple colouration of midribs. P levels in leaf laminae were 0.05 $\mu\text{g/mg F.W.}$ for ethanol soluble and 0.11 $\mu\text{g/mg F.W.}$ for

insoluble P as compared with 0.15 and 0.17 $\mu\text{g/mg}$ for plants grown normally in potting compost.

To test the effect of removing leaves from the plant, groups of leaves averaging 4.0 cm in width were excised and the cut petioles placed in Vickery's solution. The leaves were maintained alongside a similar batch of leaves remaining on the plants. After 10 days these latter leaves had an average width of 11.0 cm, the excised leaves being 4.9 cm. P contents were: control leaves—ethanol soluble 0.41 $\mu\text{g/mg}$, insoluble 0.36 $\mu\text{g/mg}$; excised leaves—ethanol soluble 0.41 $\mu\text{g/mg}$, insoluble 0.22 $\mu\text{g/mg}$.

Attempts to raise P levels. The toxicity of several salt solutions for Chinese cabbage plants was tested by placing the washed roots of whole plants into the aerated solution and observing the leaves for gross wilting or collapse of interveinal areas. At the 0.1 M level, solutions of K_2SO_4 or KH_2PO_4 regularly caused wilting of leaves after a few hours; 0.03 M KH_2PO_4 was the highest level that caused no obvious damage to the plant over periods of a few days.

The effects on P levels of placing the roots of whole plants in 0.03 M KH_2PO_4 are given in Table 2. These results suggest that at best one could expect to double or treble the level of soluble P.

We conclude from the results presented in this section that it would be impractical to carry out effective "chase" type experiments with TYMV in Chinese cabbage leaves with P^{32} -labelled phosphate.

The Time Course of Labelling of TYMV Nucleoprotein with P³²

We have attempted to determine whether or not TYMV nucleoprotein is "turning over" by determining the increase in amount of nucleoprotein in the leaves, together with the increase in the specific activity of the virus RNA P and the soluble and insoluble leaf P as a function of the time after labelling.

Twelve young Chinese cabbage plants that had been inoculated with TYMV 12 days previously were trimmed to two well-expanded systemically infected leaves. The roots were washed and placed in carrier-free P³²-labelled orthophosphate (approximately 1 mc in 1 ml per plant). These plants were maintained in the glasshouse; at various times after the leaves had been placed in the phosphate solution, disk samples were taken.

One no. 8 cork borer disk was taken from each half leaf, and from the center of these disks a no. 2 borer disk was taken. The disks were weighed, and the smaller disks were extracted in hot ethanol preparatory to P estimations and radioactivity determinations. The larger disks were used to isolate the nucleotides from virus RNA by the serological-chromatographic procedure. The specific activity of the nucleotides was determined, and from this the specific activity of virus P was calculated. The in-

TABLE 2

INCREASE IN PHOSPHORUS LEVEL^a IN CHINESE CABBAGE LEAVES AFTER ROOTS OF INTACT PLANTS HAD BEEN PLACED IN KH₂PO₄ SOLUTIONS

Leaf size	P fraction	Roots in water, 29 hr	Roots in 0.03 M KH ₂ PO ₄ , 29 hr
Small	Ethanol soluble	0.29	0.57
	Ethanol insoluble	0.49	0.45
Large	Ethanol soluble	0.15	0.39
	Ethanol insoluble	0.32	0.37

^a Phosphorus content expressed as micrograms per milligram fresh weight.

crease with time of virus P per unit fresh weight of leaf was also obtained from the amounts of nucleotides in the serological precipitates.

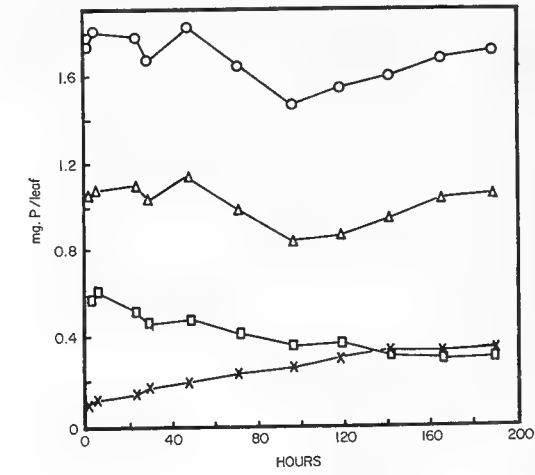


FIG. 1. Phosphorus content of Chinese cabbage leaves infected with TYMV, expressed as milligrams P per leaf. (Same experiment as Fig. 2.) Δ — Δ , ethanol-soluble P; \times — \times , TYMV P; \square — \square , nonvirus P, insoluble in ethanol (calculated by subtracting virus P from total insoluble P); \circ — \circ , total P (ethanol soluble plus ethanol insoluble).

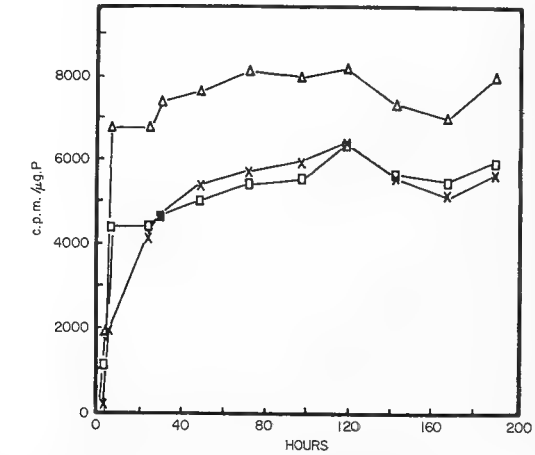


FIG. 2. Changes in specific activities of P in Chinese cabbage leaves infected with TYMV, following uptake of P³² labelled orthophosphate through the roots. (Same experiment as Fig. 1.) Roots were placed in the labelled solution at time 0. Δ — Δ , ethanol-soluble P; \times — \times , TYMV P; \square — \square , nonvirus P, insoluble in ethanol (calculated by difference from the specific activities and amounts of total insoluble-P and virus P).

Figure 1 shows the changes with time in levels of total P, ethanol soluble P, virus P, and nonvirus insoluble P, expressed as milligrams of P per leaf calculated as described under Methods. Figure 2 gives the change in specific activity of the three classes of P with time. Both ethanol soluble and nonvirus insoluble P had reached almost full specific activity at 5 hours. The specific activity of virus P rose somewhat more slowly. The specific activities of both virus and nonvirus insoluble P levelled off at a specific activity substantially below that of the ethanol soluble P. The fact that the level is about the same for virus and nonvirus insoluble P in this experiment is coincidental, as in another test in which older plants were used the nonvirus insoluble P reached a substantially higher level than the virus P. The nonvirus insoluble P as estimated here would include host RNA, but it is not known what proportion of other substances, for example polyphosphates, were present.

In these experiments specific activity of virus P was estimated on the eluted mixed nucleotides after chromatography, in isopropanol ammonia, of neutralized alkaline digests of specific precipitates. As a check that the radioactivity measured came from TYMV RNA, aliquots of nucleotides samples were subjected to electrophoresis on paper at pH 3.5. Approximately 90% of the radioactivity put on the paper was recovered from the areas of paper where the four nucleotides were expected. The radioactivity was present in ratios characteristic for the bases in TYMV RNA.

Time Course of Labelling of Virus Protein and Nucleoprotein with S^{35}

Twenty-four Chinese cabbage plants that had been inoculated 13 days previously with TYMV were trimmed to two well-expanded systemically infected leaves. The plants were arranged in groups of three, with equilization as far as possible of the variation in plant size to give comparable sets. The washed roots of the plants were placed in water containing S^{35} -labelled sulfate (approximately 0.7 mc in 1 ml, per

plant). The six leaves from a group of 3 plants were harvested at various times, the midribs removed, and TYMV prepared by the pH 4.8 procedure, with two cycles of high and low speed sedimentation. Empty protein shells and B_1 virus nucleoprotein were then isolated by two cycles of density gradient sedimentation in cesium chloride solutions.

Nitrogen determinations were carried out on the final dialysed preparations. Aliquots of these were then mixed with an excess of a B_1 rabbit antiserum. The washed specific precipitates were transferred to planchettes for the measurement of radioactivity. Figure 3 shows the time course of labelling for virus protein and nucleoprotein. Separate groups of 3 plants were used to determine each pair of points in Fig. 3. There was some variation in the total radioactive sulfur taken up by the harvested leaves (0.36 to 1.0×10^7 cpm per milliliter of expressed sap). To allow for this variation, the data in Fig. 3 have been standardized to cpm/ 10^7 cpm per milliliter of sap.

We do not have proof that the radioactivity measured in this way was in amino acids in peptide linkage in virus protein. However, the following evidence (in addition to the fairly rigorous virus isolation procedure used) suggested that most of the radioactivity was in fact in virus protein. Tests were made on virus components from leaves harvested 24 hours after labelling. Aliquots of the preparations were treated for 30 minutes at room temperature in the following ways: saturated urea, saturated sodium sulfate, and 5% mercaptoethanol at pH 7.6; none of these treatments rendered dialysable any of the radioactivity associated with either the empty protein shell fraction or the B_1 nucleoprotein.

When virus protein was precipitated by the addition of ethanol to 50% (v/v) at pH 7.0, all the radioactivity in both the virus protein and nucleoprotein preparations appeared in the denatured protein precipitates. Aliquots of these precipitates, equalized with respect to amount of protein nitrogen, were hydrolyzed in 6 N HCl and chromatographed in phenol-water followed by bu-

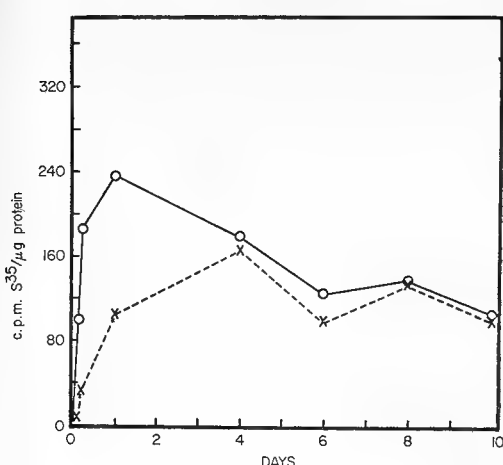


FIG. 3. Changes in specific activities of empty protein shells and B₁ virus nucleoprotein with time, following uptake of S^{35} labelled sulfate through the roots. ○—○, protein shells; X---X, B₁.

tanol-acetic acid in the second dimension. Amino acid spots were located by spraying with ninhydrin. The cysteic acid, methionine, and methionine sulfoxide spots were cut out and eluted in 50% ethanol.

The ratios of radioactivity in a fraction from virus protein, to that in a similar fraction from protein of virus nucleoprotein (for equal weights of protein nitrogen) were: whole digest, 2.4; cysteic acid, 2.5; methionine, 2.2; methionine sulfoxide, 2.6. Since the amino acid composition of both classes of protein is known to be very similar, the close agreement between the ratio of radioactivities in the sulfur-containing amino acids and their derivatives to that in the whole digest, suggests that the virus protein was in fact more heavily labelled (by a factor of about 2.5 in this particular preparation) than the nucleoprotein.

Labelling of Minor Nucleoprotein Fractions with S^{35}

In previous experiments reported briefly (Matthews, 1960), we found that over periods of a few hours' labelling of plants with S^{35} sulfate, the TYMV fractions became labelled in the order: T B₀₀₀ B₀₀ B₀ B₁ B₂.

In the experiment summarized here we followed the time course of labelling in the same set of labelled plants for a period of

several days starting 16 days after inoculation. At each time of sampling leaf disks were taken from the labelled plants and unlabelled comparable infected leaf was added for the virus preparations. Practical limitations on quantities meant: first, that only two cycles of density gradient fractionation could be carried out; secondly, that we were unable to obtain sufficient B₀₀₀ for analyses; thirdly, that we had to use optical density measurements (with an empirically determined factor) for estimating amounts of the minor fractions for specific activity measurements. Thus in the results summarized in Table 3, there was likely to have been larger error in the specific activity determinations for the minor fractions than for T and B₁.

As in previous tests T became labelled very much more rapidly than B₁ nucleoprotein. However, the specific activity of T did not fall to the level of B₁ as rapidly as in the experiment of Fig. 3. This might be explained by differences in the rate of virus increase in the two experiments. In the experiment of Fig. 3 virus nucleoprotein content of the leaves rose steadily, increasing about fourfold. In the experiment of Table 3 virus content rose about 2½-fold from 0 to 91 hours and thereafter remained more or less constant.

The last (200 hour) sample in Table 3 was prepared from the remains of the labelled leaves without the addition of carrier. There was insufficient material for the isolation of B₀₀ and B₀. However, since the other three fractions had virtually the same specific activities at this time, it is reasonable to assume that by 200 hours all fractions were uniformly labelled.

If, as has been suggested (Matthews, 1960), these minor fractions were stages in the assembly of B₁ from T, then they should have attained a specific activity close to that of T in a period of a few hours, since from the observed rate of increase in amount of B₁ they should have been used up many times over in a few hours (Table 3). Approximate calculations from the rate of increase in B₁ in this experiment (and assuming the amount of each of these fractions was 3% of B₁) show that the specific

TABLE 3

SPECIFIC ACTIVITIES^a OF TYMV FRACTIONS FROM PLANTS SUPPLIED WITH S^{35} -LABELLED SULFATE

Time after labelling begun (hours)	Fraction				
	T	B ₀₀	B ₀	B ₁	B ₂
5	630	177	128	45.0	44.0
17	796	333	274	216	147
24.5	645	354	416	229	210
41.0	676	317	308	286	284
49.5	740	187	—	307	244
91.0	761	286	—	274	285
127	618	338	304	268	119
176	500	292	256	231	217
200	310	—	—	285	296

^a Expressed as counts per minute per microgram protein.

activity of B₀₀, B₀, and B₂ should have reached virtually the same specific activity as T well before the 17-hour sampling time.

THEORETICAL TREATMENT OF THE RELATION BETWEEN VIRUS PROTEIN AND NUCLEOPROTEIN

In the following discussion we assume that both the virus protein shell and the virus nucleoprotein are assembled from small pre-formed protein subunits. If we allow the possibility that the completed protein shells of either the protein or the nucleoprotein may break down *in vivo* to the subunits (turnover) then there are a large number of possible relationships between these two main classes of virus particles. Some of the possibilities are summarized diagrammatically in Fig. 4. There is a whole series of further possible relationships, not shown in Fig. 4, which may be termed "cyclic conditions." For example, the virus nucleoprotein could be made both from empty protein shells and directly from the subunits.

Although there is a plethora of models to consider, mathematically all are of the same basic form, either involving "turnover" of virus particles (reversible reactions) or not (one-way reactions), and the latter is merely a special case of the former.

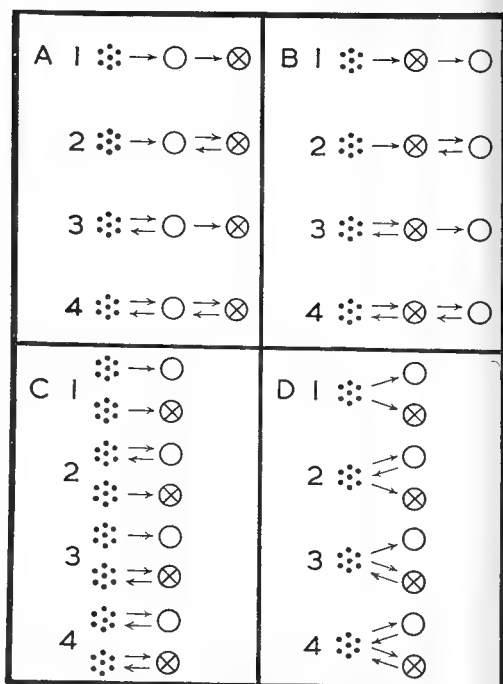


FIG. 4. Some possible relationships between virus protein precursor subunits (dot pattern), empty virus protein shells (O), and virus nucleoprotein (X). A. Empty shells precursor of virus nucleoprotein. B. Empty shells postcursor of virus nucleoprotein. C. Empty shells not a precursor of virus nucleoprotein, and made from a different pool of protein subunits. D. Empty shells not a precursor of virus nucleoprotein, but both made from same pool of subunits.

For a complete specification, we allow each virus particle, when made, to have a probability p of remaining locked up, and a probability q ($= 1 - p$) of breaking down to its immediate precursor, at a random rate per unit time. The virus population itself is growing, the observed growth curve being described by the function $V(t)$. Let $V_1(t)$ be the total amount of locked-up virus at time t , $V_2(t)$ the remainder which is turning over at rate r per unit time, and let the amount of virus actually made in the interval t to $t + dt$ be $U(t) dt$. This will be larger than the observed increase $dV(t)$ because of the random breakdown of particles made at earlier times.

If virus growth starts at $t = 0$, then

time t ,

$$V_1(t) = \int_0^t pU(\theta) d\theta$$

$$V_2(t) = \int_0^t qe^{-r(t-\theta)}U(\theta) d\theta$$

Therefore

$$V(t) = \int_0^t (p + qe^{-r(t-\theta)})U(\theta) d\theta \quad (1)$$

Differentiating with respect to t ,

$$\begin{aligned} \frac{dV(t)}{dt} &= pU(t) - rqe^{-rt} \int_0^t e^{r\theta}U(\theta) d\theta \\ &\quad + qe^{-rt}e^{rt}U(t) \\ &= U(t) - rV(t) + rp \int_0^t U(\theta) d\theta \\ &\quad \text{[from Eq. (1)]} \end{aligned}$$

Differentiating again, we obtain

$$\frac{dU(t)}{dt} + rpU(t) = \frac{d^2V(t)}{dt^2} + r \frac{dV(t)}{dt} \quad (2)$$

an ordinary differential equation which may be solved for $U(t)$, given $V(t)$. Two special cases of Eq. (2) are

$$\text{No turnover } (p = 1): U(t) = \frac{dV(t)}{dt} \quad (2a)$$

Complete turnover ($p = 0$):

$$U(t) = rV(t) + \frac{dV(t)}{dt} \quad (2b)$$

If now radioactivity is introduced into this system from time t_0 and the immediate precursor of the virus has specific activity following the function $R(t)$, ($t > t_0$, $R(t_0) = 0$) then the activity of the virus at time $t(>t_0)$ will be

$$Q(t) = \int_{t_0}^t (p + qe^{-r(t-\theta)})R(\theta)U(\theta) d\theta \quad (3)$$

since of the amount of virus $U(\theta) d\theta$ made in $(\theta, \theta + d\theta)$ with activity $R(\theta)$, only $pU(\theta) d\theta + qe^{-r(t-\theta)}U(\theta) d\theta$ remains not broken down at t .

The specific activity (S.A.) of the virus at time t is $Q(t)/V(t)$.

For the case of no turnover ($p = 1$):

$$Q(t) = \int_{t_0}^t R(\theta) dV(\theta) \quad (3a)$$

For the case of complete turnover ($p = 0$)

$$Q(t) = e^{-rt} \int_{t_0}^t e^{r\theta}R(\theta)U(\theta) d\theta \quad (3b)$$

where $U(\theta)$ is given by Eq. (2b).

In practice, use of Eqs. (2) and (3) to fit models for virus synthesis to the observed activity data involves knowledge of $V(t)$ and $R(t)$. For TYMV, growth curves for the whole virus have been fitted very well by the logistic

$$V(t) = a/1 + be^{-ct}$$

which is derived from the differential equation

$$dV/dt = bV - bV^2/c$$

the first term representing exponential growth and the second a competition effect. This form is, however, very difficult to handle mathematically. In the calculation of $V(t)$ from the experimental data, the logistic curve has been approximated by an initial exponential phase, then a straight line phase, and finally a negative exponential phase to fit the flattening at the top of the curve. For all practical purposes this approximation is virtually indistinguishable from the original curve.

The form of the uptake curves of radioactivity by precursor pools in the leaf is unknown. If an observed pool (e.g., ethanol-soluble P) was being tested as a virus precursor, its S.A. curve was approximated, in the interests of simplicity in the calculations, by a succession of straight lines. If the possibility of a unknown pool being the precursor was being considered (e.g., virus protein subunits), its proposed S.A. curve was treated in the same manner.

TESTS ON THE EXPERIMENTAL DATA WITH CERTAIN MODELS

P^{32} Labelling of B_1

Table 4 records the specific activities for B_1 P expected on various assumptions for the data of the experiments summarized in Figs. 1 and 2.

Our data eliminate the hypothesis on which column B is based, namely, B_1 P originating from P with the specific activity of ethanol-soluble P as immediate precursor without turnover. However, both columns D and E agree reasonably well with the experimental values. Thus we cannot determine from present data whether or not significant "turnover" of virus RNA P takes place. Nevertheless, we favor the hypothesis that the immediate precursor of B_1 P has a different specific activity than the total ethanol-soluble P in the early stages after label is introduced and that B_1 has no significant turnover (column E). In the first few hours after labelling it is most unlikely that the small molecular weight P compounds in Chinese cabbage leaves are uniformly labelled. There is some evidence from other plant systems to support this view. Thus R. L. Bielecki (personal communication) has followed the changes in

specific activity of phosphate esters in potato pieces after a brief period in which the pieces were allowed to take up P^{32} -labelled orthophosphate. Nucleotide di- and triphosphates and hexose phosphates became labelled much more rapidly than inorganic phosphate. For example, at 30 minutes the specific activity of ATP was about 30 times that of inorganic phosphate. Bielecki concluded that it probably takes 12–72 hours for the specific activity of phosphate esters and inorganic phosphate to come to equilibrium.

S^{35} Labelling of T and B_1

A common feature of our labelling experiments with S^{35} (e.g., Fig. 3 and Table 3) has been a rapid rise in the specific activity of T followed by a decline into a substantially lower level over a period of days. With no suitable micro method available for the estimation of sulfur, we have no informa-

TABLE 4
SPECIFIC ACTIVITIES^a EXPECTED ON CERTAIN MODELS FOR B_1 IN THE
EXPERIMENT OF FIGS. 1 AND 2

Time (t) (hours)	A	B ^b	C ^b	D ^b	E ^c
	Observed B_1	$R \rightarrow B_1$	$R \rightleftharpoons B_1$ ($r = 0.2$; $p = 0.1$)	$R \rightleftharpoons B_1$ ($r = 0.3$; $p = 0.07$)	$R' \rightleftharpoons B_1$
1½	142	30	146	181	178
5	1880	308	1310	1640	1850
23½	4090	1980	4110	4150	4080
29½	4600	2370	4400	4420	4380
48	5380	3320	5090	5120	5070
71	5660	4160	5660	5680	5630
96	5900	4730	6110	6120	5990
118	6460	5100	6390	6280	6270
141	5560	4730	5820	5820	5720
165	5120	4620	5610	5620	5520
189	5600	5330	6420	6430	6320

^a Expressed as counts per minute per microgram phosphorus.

B_1 growth curve was taken as linear from 0.098 mg B_1 P per leaf at $t = 0$ to 0.242 at $t = 72$, then a negative exponential following the equation

$$V(t) = 0.242 + 0.132 (1 - e^{-0.015t})$$

Parallel sampling variation occurred over the last four times of sampling in all P fractions (see Fig. 2). Calculated figures for these times in columns B–E in the table were adjusted arithmetically on the assumption that the reservoir remained constant at 8000 cpm.

^b R (= ethanol-soluble P) taken as going linearly from 0 to 6660 cpm at 5 hours, to 8000 cpm at 72 hours, flat thereafter.

^c R' (= hypothetical reservoir of P for virus synthesis) taken as going linearly up to 40,000 cpm at 5 hours, down to 8000 cpm at 8 hours, flat thereafter.

tion on the time course of specific activity of total soluble sulfur compounds. However we suggest the following tentative explanation for the rise and fall in specific activity of T: that a pool of soluble sulfur compounds from which T is made becomes labelled rapidly; that there is another class of sulfur compounds in the leaf containing the same order of amount of sulfur, that equilibrates slowly over a period of several days with the rapidly labelled sulfur, slowly lowering the specific activity of the sulfur compounds from which T is synthesised.

In the light of the labelling data in Fig. 3, certain of the models in Fig. 4 can be eliminated by inspection. These are: B1-4 where B₁ is a precursor of T; and D1 and D3 where T is made irreversibly from the same pool of subunits as B₁.

Any of the models C1-4, where T and B₁ come independently from different pools of

precursor, could be made to fit the data with suitable choice of parameters. Although these possibilities cannot be dismissed, we will not consider them further at this stage since simpler models will fit the data available.

In Table 5 we have considered three possibilities (A1 or A3, A2 or A4, and D2 of Fig. 4). The hypothesis that B₁ arises from T (and no other source) without turnover appears to be eliminated. On this hypothesis the specific activity of B₁ is too low at the early times (column D, Table 5).

The calculated data of both columns F and G + H in Table 5 fit reasonably with the experimental values. Thus we cannot distinguish, with the data available, between the models where B₁ arises from T with turnover (A2 and A4 of Fig. 4) and that where T and B₁ both arise from a common reservoir, T with turnover and B₁ without. Presumably the D4 model of Fig. 4 would also fit the data.

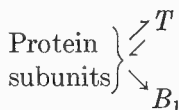
TABLE 5
SPECIFIC ACTIVITIES^a EXPECTED ON CERTAIN MODELS FOR T
AND B₁ IN THE EXPERIMENT OF FIG. 3

A Time (<i>t</i>) (hours)	B Amount of component (mg/leaf)		C Specific activities found		D ^b	E ^b	F ^b	G H	
	T	B ₁	T	B ₁	Specific activity of B ₁ T → B ₁ (A1 or A3 of Fig. 4) with <i>p</i> = 1	Specific activity of B ₁ T ⇌ B ₁ (A2 or A4 of Fig. 4) with <i>p</i> = 0.5; <i>r</i> = 0.1	Specific activity of B ₁ T ⇌ B ₁ (A2 or A4 of Fig. 4) with <i>p</i> = 0.083; <i>r</i> = 1.2	For D2 of Fig. 4 ^c	
								Reservoir ⇌ T (<i>p</i> = 0; <i>r</i> = 0.1)	Reservoir → B ₁ (<i>p</i> = 1)
2½	0.61	0.85	101	7	2	4	13	45	9
5	0.52	1.04	189	36	9	14	36	164	35
12	—	—	—	—	36	56	73	283	81
24	0.56	1.47	236	108	72	96	100	246	107
48	—	—	—	—	111	130	130	209	132
72	—	—	—	—	130	143	144	184	143
96	0.72	2.67	177	157	135	140	144	160	145
144	0.71	2.95	126	100	137	135	144	127	143
192	0.78	4.13	139	136	135	135	144	126	141
240	0.91	4.04	106	100	133	135	141	126	139

^a Expressed as counts per minute S³⁵ per microgram virus protein per 10⁷ cpm per milliliter of sap.
Growth curves for T and B₁ were approximated from the experimental curves by straight lines as follows:
T: From 0.514 mg protein per leaf at t = 0 to 0.917 at t = 240 hours.
B₁: From 0.96 mg protein per leaf at t = 0 to 2.40 at t = 72; then to 4.08 at t = 240 hours.
^b T taken as going linearly up to 260 cpm at 7 hours, down to 120 at 144 hours, and flat thereafter.
^c Common reservoir of precursor subunits taken as going linearly to 750 at 5 hours; 250 at 10 hours; 222 at 24 hours; 126 at 120 hours.

CONCLUSIONS

For the relation between T and B₁ we favor the following model as being the simplest that is consistent with the observed rates of labelling with S³⁵.



This model would also be consistent with our favored interpretation of P³²-labelling of B₁, namely that the RNA in completed virus has no significant turnover. On this view the empty protein shell might be a reversible "mistake" in virus assembly in which the protein subunits pack to form a shell in the absence of RNA.

The data on S³⁵-labelling of the minor nucleoprotein fractions appear to rule out the possibility proposed earlier (Matthews, 1960) that these components are stages in the assembly of B₁ from T. This conclusion would again be consistent with the model suggested above. If B₁ is formed independently of T, then the minor nucleoprotein fractions might, on the basis of their labelling behavior, be stages in the assembly of B₁. However, from the evidence available so far it is just as probable that B₁ is formed by the packing of protein subunits around the nucleic acid core. The minor nucleoprotein fractions might then represent classes of recently formed particles which vary in the ease with which a proportion of their RNA complement is lost during the isolation, or in the case of B₂ the loss of some other property necessary for infectivity.

To obtain more conclusive evidence it will be necessary, as far as RNA labelling is concerned, to identify the true precursor reservoirs for the components of the virus RNA (presumably nucleoside 5'-triphosphates) and to follow the specific activity of these along with that of the virus RNA, preferably by the use of C¹⁴ labelling in one or more bases.

For the protein component, again a more specific label than S³⁵ would be preferable, for example C¹⁴-labelled valine. As with the kinetics of RNA labelling, further progress would be made by knowledge of the rate at which the true immediate precursor reservoir for protein synthesis becomes labelled. No one has yet identified the occurrence of free virus protein subunits in infected leaves. If these occur in quantities that can be handled experimentally, then knowledge of the time course of incorporation of, say, C¹⁴-valine into free valine, the protein subunits, T and B₁ might give conclusive evidence about the relation between these virus components.

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B. HydraV.B.1 Migration of ^{14}C -Labeled Cnidoblasts

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ALTHOUGH the morphogenetic movements of cells constitute a major phase of the process of development, the migration of only a few cell types has been investigated extensively. Lack of apt methodology perhaps accounts for the relative scarcity of research in this area of morphogenesis. A series of studies has been undertaken concerning the migration of the *Hydra* cnidoblast, the cell which forms the nematocyst ("stinging organoid") [1]. This system was chosen because it is possible to selectively remove the nematocysts from the cnidoblasts of the tentacles by simply feeding the *Hydra*; for, on feeding, the nematocysts are discharged, eliminated from the tentacles, and replaced by new cnidoblasts migrating from the body tube. Methods have been devised making it possible to follow the migration of the cnidoblast from its site of origin, the body tube, to its main site of function, the tentacles, by (1) a chemical assay for hydroxyproline, an imino acid found to be specifically localized within the cnidoblast as part of the nematocyst capsule [3], and (2) radioautography. The present paper describes the second of these methods. The migration can be visualized and measured by selectively labeling the developing cnidoblasts with $^{14}\text{CO}_2$; after these cells are labeled, the direction and rate of their migration can be followed by radioautography.

Groups of 10 one-day starved *Hydra littoralis*, obtained from laboratory grown mass cultures [5], were placed in a 15 ml single-armed Warburg vessel; the total volume of culture solution was brought up to 2 ml. After 0.1 ml $\text{Na}_2^{14}\text{CO}_3$ (about 10–20 μC) was placed in the side arm, a few drops of concentrated sulphuric acid was added to this side arm to generate $^{14}\text{CO}_2$; the flask was immediately sealed. (Neutralized $\text{Na}_2^{14}\text{CO}_3$ could also be added directly to the solution bathing the *Hydra*.) The *Hydra* were removed from their radioactive environment after the appropriate incubation times and were washed 3 times in clean culture solution.

The *Hydra* were prepared for radioautography in the following manner: a moist membrane filter (Millipore, 1 inch diam.) was placed on a flat sintered glass filter set on a Buchner suction flask. The *Hydra* were placed in a small drop of water on the moistened filter disc. When the animals were completely relaxed with their tentacles extended, the flask was immediately evacuated so that the outstretched *Hydra* were drawn against the filter disc. In order to prevent the contraction of the *Hydra*, they were killed by heating for one minute with an infra-red lamp. While the vacuum pump was still operating, the *Hydra* and filter paper were washed with distilled water to remove any free soluble radioactive isotopes. The washed filter disc was glued on a 1" \times 3" glass slide, and then dried under the infra-red lamp for 5 minutes. Radioautographs were made by the apposition method using NTB₂.

nuclear track plates (Kodak). The plates were exposed to the labeled *Hydra* for 2 to 4 weeks.

Radioautographs of animals exposed to $^{14}\text{CO}_2$ for 16 hours (Fig. 1) revealed that most of the radioactivity was confined to small but discrete "loci" throughout the upper two-thirds of the body tube. Few, if any, of these loci were present in the tentacles. If, on the other hand, *Hydra* were allowed to starve in a non-radioactive environment for 48 hours after being exposed to $^{14}\text{CO}_2$, many of the loci would appear in the tentacles (Fig. 2). This migration of the labeled loci from the body tube to the tentacles suggests that the cnidoblasts, which are making nematocysts while in the body tube, migrate to the tentacles where the nematocysts are to be used. Actual counts of radioactivity demonstrated that during the 2 days after the *Hydra* were removed from the $^{14}\text{CO}_2$ environment, the proportion of the activity in the tentacles as compared to the activity in the body tube increased many fold. A radioautograph of the tentacles and hypostome of a similarly treated animal demonstrated the dense concentration of radioactive nematocysts in the tentacles, and the probable presence of migrating cnidoblasts in the hypostome region while in the midst of their journey (Fig. 3).

Experiments have been carried out in which the *Hydra* were exposed to $^{14}\text{CO}_2$ for 16 hours during a period when a bud was just beginning on each of the animals. The bud primordia were allowed to complete their development during the next 48 hours in a non-radioactive environment, and a radioautograph was made of buds after they detached. As shown in Fig. 4, the batteries of the bud's tentacles are strongly labeled, as are also the cnidoblasts in the body. Since the bud developed from the prelabeled parent, it apparently did not make all its own nematocysts but was given a large share of nematocysts preformed by its parent.

In an experiment in which an non-radioactive hypostome with a ringlet of tentacles was grafted onto a ^{14}C -labeled body tube of another animal, the radioactive cnidoblasts migrated from the body tube and were deposited along the tentacles. Thus, even tissue from another individual *Hydra* can be invaded by cells from the body tube.

It is of interest to consider the possible mechanisms whereby the cnidoblasts incorporate most of the $^{14}\text{CO}_2$ in the *Hydra*. Two-dimensional chromatographic analysis of the acid hydrolyzed animals demonstrated that most of the radioactivity is found in glutamic and aspartic acids. These amino acids probably arise from the $^{14}\text{CO}_2$ incorporated (by means of the Krebs cycle) into α -ketoglutaric acid and oxaloacetic acid by transamination. This finding implies that the Krebs cycle operates in *Hydra* as it does in other organisms, and can function to provide amino acid intermediates for protein synthesis. Preliminary kinetic studies indicate that the ^{14}C is first incorporated into the amino acid pool (cold-trichloroacetic acid soluble) which is rapidly converted into protein (hot-trichloroacetic acid precipitable). Thus, the radioautographs, the presence of ^{14}C -labeled glutamic and aspartic acids, and the preliminary kinetic incorporation data make it appear that the cnidoblasts incorporate $^{14}\text{CO}_2$ because these cells are active in synthesizing protein in the one-day starved *Hydra*. Direct chemical measurements for hydroxyproline also indicate that the cnidoblasts of one-day starved *Hydra* are actively synthesizing hydroxyproline-containing proteins [2]. Also, electron micrographs of cells from starved *Hydra* demonstrate that the cnidoblasts are rich in RNA-containing endoplasmic reticulum, indicative of protein synthesis [6]. Further experiments were preformed in which germ cells, in addition to the cnidoblasts, were induced to form in the starved *Hydra*; thus the newly synthesized germ cell proteins should also incorporate $^{14}\text{CO}_2$. In these experiments, the $^{14}\text{CO}_2$ was incorporated into the testes of male *Hydra* (Fig. 5) and the ovary of the female (Fig. 6). The $^{14}\text{CO}_2$ incorporation into newly differentiated *Hydra* gonads is especially interesting in light of Loomis' remarkable demonstration

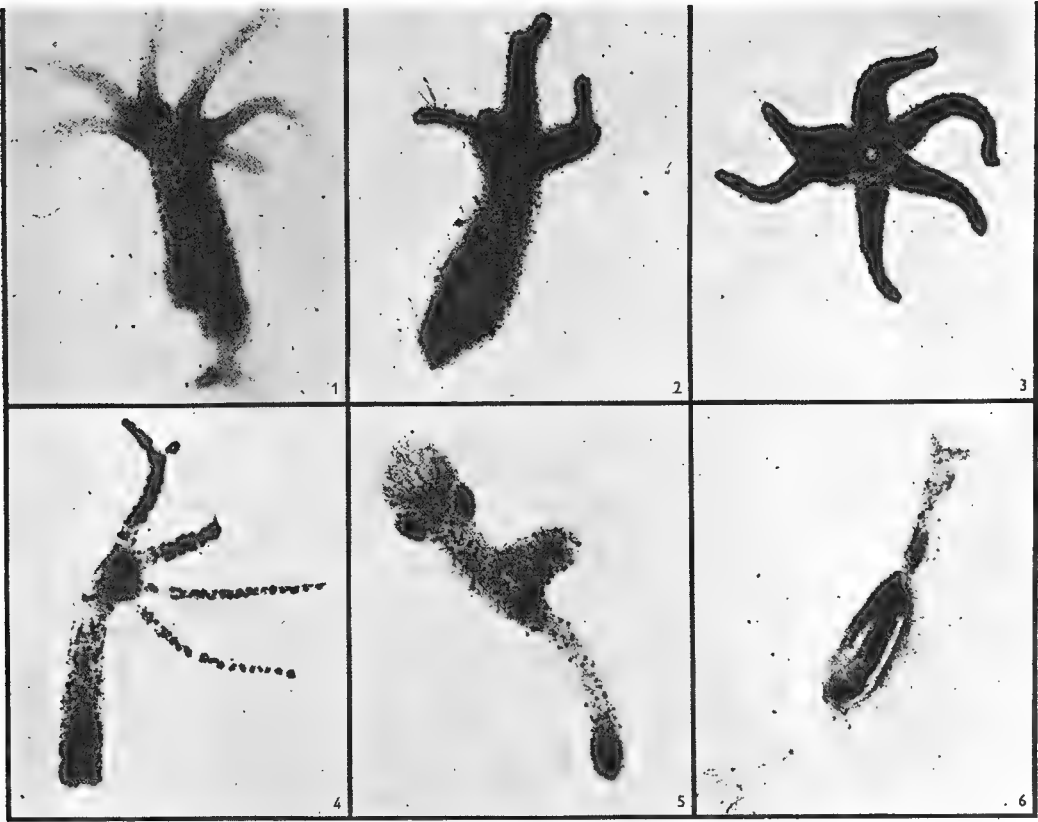


Fig. 1.—Radioautograph of a *Hydra* exposed to $^{14}\text{CO}_2$ for 16 hours.

Fig. 2.—Radioautograph of a *Hydra* exposed to $^{14}\text{CO}_2$ for 16 hours, and subsequently placed in a non-radioactive environment for 48 hours.

Fig. 3.—Radioautograph of tentacles and hypostome of *Hydra* treated similarly to the *Hydra* described for Fig. 2.

Fig. 4.—Radioautograph of bud developed in unlabeled environment from parent exposed to $^{14}\text{CO}_2$ for 16 hours.

Fig. 5.—Radioautograph of a body tube of a sexually differentiating male *Hydra* exposed to $^{14}\text{CO}_2$ for 16 hours. Note two labeled testes on upper part of tube.

Fig. 6.—Radioautograph of a sexually differentiating female *Hydra* exposed to $^{14}\text{CO}_2$ for 16 hours. Note heavily labeled ovary on middle part of animal.

that the pCO_2 of the environment controls the extent of sexual differentiation in *Hydra* [4].

Since Trembley's classical studies in 1740, *Hydra* have often been used as an experimental animal in investigations concerned with regeneration, asexual reproduction, polarity, grafting, induction, etc. The tentacles, which are usually associated with the anterior portion of the animal, have been used as a morphological marker in nearly all of these studies. The development of a tentacle, however, includes many complex cellular phenomena and it would be experimentally advantageous to investigate only one of these phenomena at a time. The radioautographic procedures discussed in this paper, plus a quantitative chemical assay for cnidoblasts [2] may provide methodology to study one major process in tentacle development—the

cellular migrations of the cnidoblast to the tentacle. Thus, by focusing on one aspect of tentacle development, we may approach previously indiscernible mechanisms involved in regeneration and related phenomena.

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V.B.2 Digestion of Protein in *Hydra* as Studied Using Radioautography and Fractionation by Differential Solubilities

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WHEN a small animal, such as *Daphnia*, accidentally swims into a *Hydra*'s tentacles, the *Hydra* instantaneously pierces the prey with its deadly penetrant nematocysts. In the body fluids oozing from the wounds caused by the nematocysts are small amounts of reduced glutathione. This tripeptide induces the *Hydra* to open its mouth and swallow the prey [14]. By capturing small organisms in this manner, the *Hydra* is able to obtain sufficient food for growth.

The mechanism by which *Hydra* assimilate ingested protein has been investigated chiefly by histological techniques. The classical work of Metschnikoff demonstrated that in a variety of coelenterates digestion occurred intracellularly [16]. Food vacuoles have been observed in gastrodermal cells by many workers. Burnett has recently provided histochemical evidence supporting the view that most digestion occurs intracellularly [4]. Soon after Metschnikoff's discoveries Greenwood pointed out that in *Hydra* the food had to be partially degraded preliminary to phagocytosis [6]. In addition, extracellular proteolytic enzymes are known to occur in the gastrovascular cavity [2, 18]. These observations have led to the inference that protein digestion is partly extracellular and partly intracellular [7].

Since digestion is a metabolic process, it would be desirable to corroborate the conclusions derived from the histological studies by evidence of a chemical nature. In the present study, utilizing primarily the techniques of radioautography and fractionation by differential solubilities, we followed the fate of ³⁵S-labeled mouse protein ingested by *Hydra*. The results were in accord with the histological evidence demonstrating that most of the protein digested by *Hydra* occurred intracellularly.

Additional data is presented concerning the rate of food-particle engulfment, the rate of excretion, and the cellular areas involved in phagocytosis. The functions of intracellular digestion are discussed.

MATERIALS AND METHODS

In feeding *Hydra* any radioactive macromolecule, two simple procedures must be used. First, the macromolecule, in this case ^{35}S -labeled protein, must be a component of some tissue which can be manipulated and ingested by the *Hydra*. Secondly, the tissue must be made acceptable to the *Hydra* by the presence of small amounts of reduced glutathione (GSH) in the environment, because in the presence of GSH the *Hydra* will swallow nearly any relatively solid object of acceptable dimensions [14].

Feeding Hydra radioactive food.—Mass cultures of *Hydra littoralis* were grown in the laboratory by the method of Loomis and Lenhoff [15]. The radioactive food consisted of ^{35}S -labeled mouse liver. The mouse was injected intraperitoneally with 3 mC of a neutralized hydrolysate of ^{35}S -labeled *T. utilis* cells (Schwarz Laboratories, Inc.), and was killed 24 hours after injection. The labeled mouse tissues were stored at -18°C .

Approximately 10–12 *Hydra* of equal size, each having one bud, were placed overnight in a Petri dish containing Versene-treated culture solution [15] so that by the following morning their basal discs were affixed securely to the bottom of the dish. It was found that when the *Hydra* adhered to a solid substratum, they were much easier to feed. Feeding was also facilitated by having an excess of culture solution in the dish so that there would always be at least 1 cm of solution above the upright *Hydra*; otherwise, in the presence of GSH, the *Hydra* would often attach their mouths to the surface of the solution rather than to the food. The *Hydra* were starved for 1 day before being fed the radioactive food.

A piece of radioactive mouse liver (ca 0.4 cm in diameter) was placed in 1 ml of 10^{-4} M solution of GSH. The tissue was cut into 15–20 small bits while being viewed under a dissecting microscope and was allowed to remain in the GSH for 5 minutes. At this time a small bit of the radioactive tissue was placed in the center of the *Hydra*'s ringlet of tentacles as close to the mouth as possible with the aid of watchmaker's forceps. As soon as this was done, one drop of the fresh GSH solution was placed near the same *Hydra*. Under these conditions the *Hydra* ingested the radioactive tissue. By following this procedure it was possible to feed over 50 *Hydra* in one half-hour.

Removal of food from the gastrovascular cavity of the Hydra.—Fractionations carried out during the first 5–6 hours after ingestion were complicated because the radioactivity was distributed in both the food within the *Hydra*'s cells and in the gastrovascular cavity. Since the structure of the *Hydra* is essentially that of a hollow tube, the gut contents were easily separated by the following procedure. Each *Hydra* was placed in a small drop of water in a flat plastic cup. At the required time, the *Hydra* were individually bisected longitudinally, using a new scalpel blade for each *Hydra*; a few drops of culture solution were placed on the bisected animals to wash some of the undigested food out of the cavity. Then each animal was individually washed three times with clean culture solution passed through a micro-pipette until

the gut was free of all visible material when viewed with a dissecting microscope at a magnification of $\times 19.5$; the washings were saved. The *Hydra* tissue, free of cavity contents, and the washings were separately fractionated and counted.

Fractionation of the tissues by differential solubilities.—Usually 10–12 ^{35}S -labeled *Hydra* had sufficient radioactivity to be fractionated and counted in a gas-flow counter. Before the fractionation, the animals were washed in distilled water. The cells were disrupted with a small glass tissue grinder. The fractionation for the different cellular chemical components was based on the methods of Roberts *et al.* [17]. Some modifications were found to be necessary in order to recover most of the radioactivity when working with such a small amount of tissue. The fractions were prepared in the following manner.

Cold TCA-soluble fraction.—The disrupted tissue was brought to 1 ml with distilled water. To this was added 1 ml of 10 per cent TCA;¹ the mixture was allowed to stand 15 minutes at room temperature and a portion (usually 1 ml) was centrifuged for 15 minutes at 10,000 *g*. When the TCA-S solution was removed from the centrifuge tube, a few particles of the TCA-I precipitate would usually dislodge and contaminate that solution. Since a few TCA-I particles might contain sufficiently high radioactivity to significantly alter the results, the following procedure was followed to obtain TCA-S free of TCA-I. The supernatant fluid was passed through a membrane filter 1 inch in diameter (Millipore) of coarse porosity, set in a "micro-analysis filter holder" (Millipore Filter Corp. XX1010000) and collected in a clean test tube. The filtered cold TCA-S fraction was always found to contain radioactive cysteine-cystine and methionine; no radioactive peptides or proteins were present as determined by paper chromatography. The filter disc was placed on a planchet and counted directly. Since this contained a part of the TCA-I material, it was saved for later calculations of the derivatives of the TCA-I fraction described below.

Cold TCA-insoluble fraction.—The TCA-I fraction was obtained by passing a portion of the remaining TCA suspension through a membrane filter. The TCA-I material retained on the filter was washed several times with 5 per cent TCA. The membrane filter was dried and the radioactivity of the material collected on it was counted. No significant self-absorption of the β -rays occurred in the fine film of radioactive material on the filter.

Total protein as alcohol-soluble and alcohol-insoluble fractions.—To the TCA-I remaining in the glass tube after the TCA-S was poured off, were added 4 ml of 80 per cent ethanol. The first few drops were added slowly and the precipitate was broken into a fine suspension with a glass rod. Next, 0.1 ml of 0.1 *N* HCl was added to the suspension. (This acid was added because, as pointed out by Roberts *et al.* [17], the quantity of Alc-S protein depends upon the pH of the alcohol solution. The largest amounts of Alc-S material were found at an acid pH.) This suspension was then placed in a 45°C water bath for 30 minutes. At the end of this period, the

¹ The following abbreviations and descriptive terminology are used: TCA, trichloroacetic acid; TCA-S, TCA soluble; TCA-I, TCA insoluble; Alc-S, TCA-I and alcohol soluble; Alc-I, TCA-I and alcohol insoluble.

The *Hydra* can be described as essentially a diploblastic tube with a *basal disc* at one end and a *mouth* surrounded by a ringlet of *tentacles* at the anterior end. The mouth is at the apex of a conical projection known as the *hypostome*. The cavity of the tube is the *gut* (alimentary canal) and is called the *gastrovascular cavity*. The inner layer of cells, or *endoderm*, is often called *gastroderm*.

suspension was passed through a membrane filter. The clear filtrate collected was the Alc-S fraction. The Alc-I fraction, which remained on the filter disc as a thin film, was washed several times with 80 per cent ethanol. The filter disc was placed on a planchet, dried, and counted.

The ^{35}S -material in the Alc-S fraction of bacteria has been described to occur chiefly as peptides [17]. An analysis of the Alc-S fraction of the *Hydra* and food tissues used here demonstrated that this fraction did not migrate on a chromatogram suspended in a butanol-formic acid solvent. Thus, as in bacteria, this fraction appears to be nonlipid and is probably entirely large peptide (and perhaps some protein). A two-dimensional chromatogram of an HCl hydrolysate of the Alc-S fraction revealed the presence of all the amino acids common to *Hydra*. The ^{35}S was distributed between cysteine-cystine and methionine, the former group having more total radioactivity. This distribution of radioactivity was similar to that of Alc-S and Alc-I fractions prepared from the mouse liver. On extraction with ether, some of the Alc-S material precipitated.

The average recovery of radioactivity in all the experiments reported in this study was between 90 and 110 per cent. Since only the radioactive sulfur was counted, no further fractionation of the Alc-I had to be carried out to remove nucleic acid. Therefore, the radioactivity could always be considered to be due to either cysteine-cystine or methionine. On analysis, the cold TCA-S material was found to contain mostly the free amino acids, while the Alc-S and Alc-I of the labeled TCA-I fraction contained large peptides (or small protein) and proteins, respectively.

Whole animal radioautography.—*Hydra* were prepared for radioautographs by a method recently described [11]. The radioautographs were made by the apposition method using 1×3 " Kodak nuclear track plates, NTB₃. In the experiments described in this paper, the plates were given less than one day's exposure.

Separation of the ectoderm and endoderm.—Radioactive *Hydra* having no buds were placed in 1.5 per cent NaCl. After five minutes, the basal disc and ringlet of tentacles were cut off with a scalpel. The remaining body tube was left in the saline solution for another ten minutes. This fifteen-minute exposure to dilute saline facilitated the subsequent separation of the ectoderm from the endoderm by means of glass microneedles. This procedure is based upon an unpublished method of C. Fulton.

Methods of expressing the fractionation data.—The results of the fractionation experiments (Figs. 2 and 3) could not be expressed meaningfully in absolute values, such as counts per minute. This is because the total radioactivity of the ^{35}S -labeled tissue fed to the different *Hydra* was not the same. Therefore, the radioactivity of the different chemical fractions was expressed in terms of percentages. In order to correctly interpret the data, the proportion of the radioactivity occurring as either amino acid (TCA-S), peptide (Alc-S), or protein (Alc-I) was expressed in two ways: (1) as the percentage of the radioactivity present either in the food remaining in the gut, or in the food taken up by the *Hydra* cells, and (2) as the percentage of the total radioactivity of the food ingested by the *Hydra*. This latter value is the sum of the radioactivity present in both the gut contents and in the *Hydra*'s cells.

When the data were expressed as the percentages of either the food within the gut or within the *Hydra* tissue considered apart from each other, these data were useful in determining the changes occurring separately in these two parts of the *Hydra*. On the other hand, the data expressed as the percentages of the *total* radioactivity

(gut contents and *Hydra* tissue) were useful in determining absolute changes in the proportions of the different chemical components.

Determination of hydroxyproline.—This imino acid was determined by a modification of the method of Stegemann [19] by Woessner [21].

RESULTS

*Rate and efficiency at which ^{35}S -labeled material was taken up by *Hydra*.*—At different intervals after feeding *Hydra* ^{35}S -labeled food, the contents of their gastrovascular cavities were removed. The different groups of washed

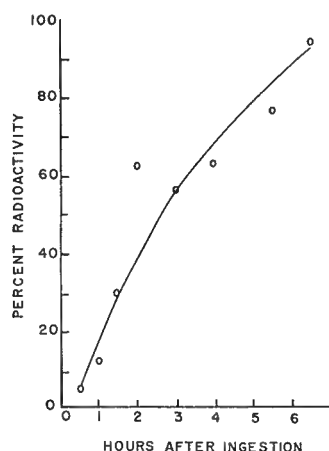


Fig. 1.—Rate at which radioactive material was taken up by the *Hydra*'s cells.

Hydra tissue and of gut contents were dried and counted. The rate at which the ^{35}S -label was taken up by the *Hydra* cells was shown by plotting the percentage of the total radioactivity (gut contents plus *Hydra* tissue) contained in the *Hydra* tissue alone at different times after ingestion (Fig. 1). At 6½ hours the *Hydra* regurgitated; therefore, the last point in Fig. 1 compares the radioactivity of the *Hydra* with that of the unused food eliminated at that time. The data indicate that the uptake of ^{35}S -labeled food by *Hydra* is relatively efficient because these animals retained over 90 per cent of the ingested radioactive food. As the figure shows, the food was taken up by the *Hydra* cells at a somewhat faster rate during the first three hours than in the latter 3½ hours. These results obviously do not tell us whether digestion occurs extracellularly or intracellularly.

Fractionation of radioactive food remaining in the gastrovascular cavity.—The data in Fig. 2, which deals with the gut contents, is expressed as either the percentage radioactivity of the gut contents (open circles), or as the per-

centage of the total radioactivity of the combined gut contents and *Hydra* cells (solid circles).

It can be seen (Fig. 2 A, solid circles) that the small proportion of free amino acids (TCA-S) present in the ingested tissue did not change significantly during the six hours following ingestion. However, when the free amino acids were calculated as the percentage of the food remaining in the gut, the proportion of the free amino acids contained in the gut to the other components of the gut increased at a linear rate to about eight-fold (Fig. 2 A, open circles). The *Hydra*'s mouth was tightly closed during this time until regurgitation occurred. Thus, the possibility of free amino acids escaping through the mouth prior to regurgitation is excluded.

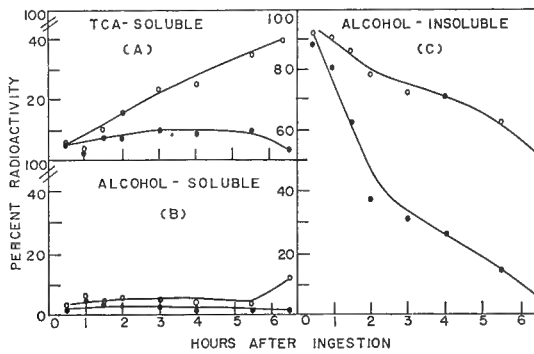


Fig. 2.—Changes in the proportions of the TCA and alcohol fractions of the radioactive food remaining in the gut following ingestion.

These results are interpreted to signify that during the first six hours following ingestion, the proportion of the free amino acids originally present in the ingested food in the gut was not augmented significantly by extracellular proteolysis. At the same time, of the radioactivity remaining in the gut, the proportion of the amino acid fraction increased due to the selective removal of larger components (shown elsewhere to be proteins) by the gastroderm. Thus, it appears that no significant hydrolysis of the food proteins to amino acids occurred extracellularly.

The fate of the Alc-I fraction of the food is shown in Fig. 2 C. The results demonstrate that the Alc-I fraction, which comprised nearly 90 per cent of the total ingested ^{35}S -label, was nearly completely removed from the gastro-vascular cavity, only 6 per cent remaining (solid circles). When the Alc-I fraction was viewed as the proportion of the undigested food in the gut (Fig. 2 C, open circles), it can be seen that the Alc-I fraction decreased at about the

same rate as the TCA-S fraction increased (Fig. 2A, open circles). This result indicates that the apparent increase in the free amino acids within the gut (Fig. 2A, open circles) was actually the result of the removal of the protein fraction (Fig. 2C, open circles).

The changes in the large peptides (Alc-S) of the gut contents were slight (Fig. 2B). This result is interpreted to signify that the ingested protein was not appreciably hydrolyzed to peptides in the gastrovascular cavity, thus lending support to the view that most of the protein was degraded intracellularly.

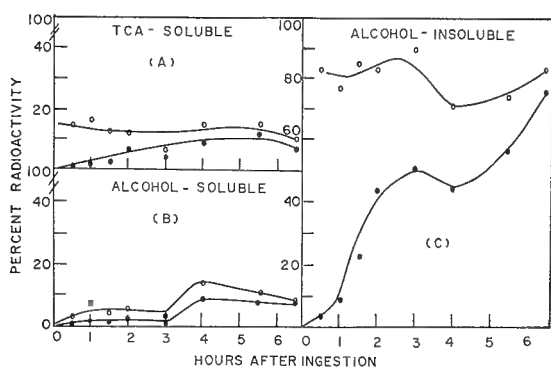


Fig. 3.

Fig. 3.—Changes in the proportions of the TCA and alcohol fractions of the radioactivity within the *Hydra's* cells following ingestion.

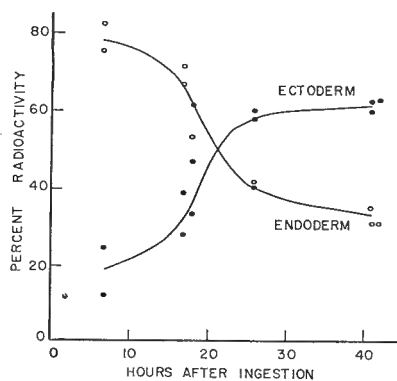


Fig. 4.

Fig. 4.—Proportions of radioactivity in the endoderm and ectoderm following regurgitation.

Fractionation of radioactive Hydra.—The data in Fig. 3 is expressed as is the data in Fig. 2, except Fig. 3 is concerned primarily with the radioactive fractions present in the *Hydra* cells. The results demonstrate that the Alc-I fraction inside the cells (Fig. 3C, solid circles) increased at nearly the same rate as the Alc-I fraction in the gut contents disappeared (Fig. 2C, solid circles). However, as shown in Fig. 3C (solid circles) a decrease in the rate of uptake was observed at four hours following ingestion. At the same time that this drop in the curve was observed, a small but significant increase in the Alc-S fraction occurred (Fig. 3B). I place special emphasis on this small increase because of results obtained from experiments related to this problem [9] (see Discussion). One possible interpretation of this discontinuity in the increase in the Alc-I would suggest that some of the intracellular Alc-I fraction was partially hydrolyzed to smaller units. Accordingly, nearly all of the radioactivity taken up by the *Hydra* cells can be accounted for by adding together the Alc-I and Alc-S fraction.

The results in Fig. 3A (solid circles) show that the TCA-S fraction of the *Hydra* cells increased slowly, always comprising about 10 to 15 per cent of the total radioactivity within the *Hydra*'s cells (Fig. 3A, open circles). These amino acids may have been taken in along with the food protein phagocytized by the gastroderm, or may have resulted from the partial hydrolysis of the radioactive protein occurring intracellularly.

The final distribution of radioactivity within the *Hydra*'s cells after regurgitation shows 80 per cent in the Alc-I fraction, 10 per cent in the Alc-S fraction, and 10 per cent in the TCA-S fraction.

Transfer of radioactivity of the food protein from the endoderm to the ectoderm.—The two *Hydra* cell layers, the ectoderm and endoderm, were separated at varying intervals following the ingestion of ^{35}S -labeled tissue, and were counted for radioactivity. The results (Fig. 4) demonstrate that for 20 hours the endodermal cells were more radioactive than the ectoderm. A relatively rapid transfer of a large share of the radioactivity to the ectoderm began at this time.

These data suggest that the endodermal cells, which engulfed the food, retained most of the ^{35}S -labeled food incompletely digested within their food vacuoles. After about one day, the labeled food proteins possibly were degraded further into molecular species which then passed on to the ectoderm where they were available for ectodermal protein synthesis. During this interval in which the labeled food protein was retained by the endodermal cells, the *Hydra* were probably using unlabeled food which was in the food vacuoles from previous meals. The slight amount of radioactivity that was present in the discarded tentacles did not affect the values presented here.

The relative absence and diffuse distribution of label from recently ingested food proteins in radioautographs of regenerated tentacles.—*Hydra* which had just regurgitated six hours after being fed ^{35}S -labeled protein were decapitated at this time and allowed to regenerate. After three days, when new tentacles had formed, the animals were killed and radioautographs were prepared (Fig. 5). Another group of *Hydra* were decapitated two days following ingestion of the ^{35}S -labeled tissue and allowed to regenerate new tentacles during the following three days. Radioautographs of the first animals (Fig. 5) demonstrate a diffuse and undifferentiated distribution of ^{35}S —in the newly regenerated tentacles. This radioautograph suggests that only a small portion of the newly ingested food was available for the formation of the regenerating cells present in the tentacles. On the other hand, the radioautograph of the second group of animals (Fig. 6) demonstrate a denser concentration and highly differentiated distribution of the ^{35}S in the tentacles. No sharp demarca-



Fig. 5.—Radioautograph of regenerated *Hydra* which was decapitated six hours following ingestion of radioactive food (see text).

Fig. 6.—Radioautograph of regenerated *Hydra* which was decapitated 48 hours following ingestion of radioactive food (see text).

tion is observed between the tentacles and body tube, as shown in Fig. 5. Therefore, this radioautograph (Fig. 6) suggests that during the two days following feeding, the ^{35}S -labeled food protein in the cells had been digested and turned over so that all the cells populating the regenerated area were significantly labeled. These results are in agreement with the data presented in Fig. 4, signifying that complete breakdown of food protein takes place about one day following ingestion.

It is interesting to note that in the latter radioautograph (Fig. 6) there exists a "metabolic ^{35}S gradient" of the type described by Child [5] from the upper portion of the body tube to the basal portion.

The synthesis of new protein for as long as two days following food ingestion.—In studies of intracellular digestion in various organisms it is very difficult to measure the synthesis of new proteins. This difficulty arises from the inability to distinguish the proteins of the food within the food vacuole from the functional proteins of the cells of the organisms. In *Hydra* this difficulty is partially eliminated because these animals contain an unusual hydroxypro-

line-rich protein which comprises about 10 per cent of their total protein [13]; this protein forms part of the nematocyst capsule. Since the food of the *Hydra* (in this case the unlabeled live brine shrimp larvae *Artemia*) was virtually free of hydroxyproline [12], it was possible to get an indication of the rate of synthesis of a particular class of proteins by determining the rate of hydroxyproline synthesis following the ingestion of unlabeled shrimp. The total

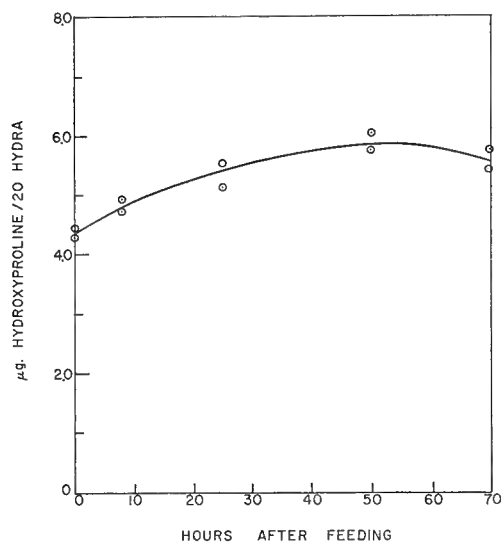


Fig. 7.—Increase in the hydroxyproline content of *Hydra* following the ingestion of unlabeled *Artemia*.

hydroxyproline can be considered to be representative only of protein and not of the free imino acid because in *Hydra* [12], as in most organisms [20], this imino acid is not found in significant concentrations in the free state. The results shown in Fig. 7 demonstrate that, following ingestion of shrimp, *Hydra* synthesize hydroxyproline for about two days, resulting in a total increase of over 30 per cent in their hydroxyproline content.

The synthesis of hydroxyproline-rich protein was gradual and occurred at a relatively constant rate for 48 hours. In contrast, the uptake of food is sudden and, under our routine laboratory procedures, occurred during a six-hour period once daily. These results can be interpreted in at least two ways: (1) the breakdown products of the food protein were converted to some storage protein which provided amino acids for the continuous synthesis of the nematocysts capsule, or (2) these amino acids were derived from the gradual breakdown of ingested food within the food vacuoles resulting from the daily feedings. The mechanism described in the latter interpretation is the more efficient one and is favored here.

Distribution of radioactivity in the Hydra's body following ingestion of la-

beled food.—The technique of whole animal radioautography was used to determine the cellular areas of *Hydra* active in taking up the radioactive food. This group of radioautographs was taken from *Hydra* fed ^{35}S -labeled mouse lung [9]; similar radioautographs were obtained from animals fed ^{35}S -labeled tissue from the other mouse organs. Fig. 8 is a radioautograph of an animal killed one hour after the ^{35}S -labeled food was ingested. The partially disrupted food is seen in clumps within the gastrovascular cavity; no radioactivity is seen in the tentacles. After three hours (Fig. 9) the radioactive material was fairly equally distributed throughout the animal, except for the tentacles and the lower region of the body tube. Fig. 10 is a radioautograph of a *Hydra* taken after it had regurgitated its undigested food six hours following ingestion; most of the ^{35}S -label was evenly distributed over the entire body except for the lower part of the body tube and the tentacles. Fig. 11 is a radioautograph of a special *Hydra* which was obtained by grafting a non-radioactive hypostome with its tentacles to the body tube of a radioactive *Hydra* which had been decapitated. A radioautograph of this "hybrid" taken after two days revealed the presence of radioactivity in the grafted tentacles.

In Fig. 10 there can be seen two non-radioactive depressions in the upper sides of the body tube which did not become labeled. These depressions correspond to two testes along the body tube of the *Hydra*. Since the testes are ectodermal, this radioautograph adds support to the data presented in Fig. 4 demonstrating that most of the ^{35}S -material was retained by the endoderm during the first day after ingestion.

The striking absence of significant radioactivity in the tentacles, as seen by the radioautographs in Figs. 8–10, could signify that tentacle endoderm does not function to any degree in taking up food. On the other hand, it might be argued that the radioactivity in the tentacles could not be visualized at these exposures because of the relative smaller size of the tentacles when compared to the body tube. This latter objection seems unlikely because definite radioautographs of tentacles were obtained from *Hydra* which had similar amounts of label; also these radioautographs were of similar exposure

Fig. 8.—Radioautograph of *Hydra* with labeled food in its gastrovascular cavity for one hour.

Fig. 9.—Radioautograph of *Hydra* with labeled food in its gastrovascular cavity for three hours.

Fig. 10.—Radioautograph of a *Hydra* which has regurgitated its undigested wastes six hours after ingestion of labeled food.

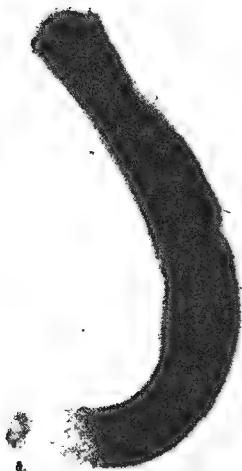
Fig. 11.—Radioautograph of a "hybrid" *Hydra* consisting of ^{35}S -labeled body tube grafted to an unlabeled hypostome and tentacles. The radioautograph was made two days after the grafting operation.



8



9



10



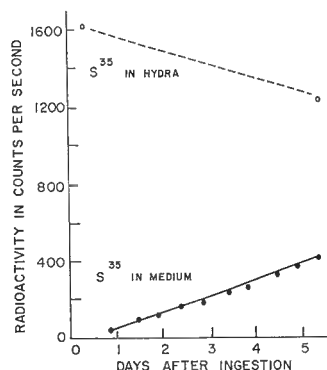
11

times (Figs. 5, 6, and 11). However, these latter groups of *Hydra* were given sufficient time to allow for the label to get into the tentacle.

Little radioactivity was observed in the lower region of the body tube even after the undigested food was eliminated (Fig. 10). It is possible that this region is not active in taking up food from the gastrovascular cavity, especially since the cells in this lower area are thought to be "older" cells [3]. However, there does seem to be some incorporation in the basal disc zone, which is thought to be relatively active in synthesizing adhesive substances [7].

Rate of discharge of label into medium following a single feeding.—The rate at which ^{35}S -compounds were released into the medium during starvation was measured by sampling the radioactivity at 12 hour intervals in the culture solution around *Hydra* which were washed after regurgitation. The experiment was carried out in the following manner. After 10 *Hydra* were fed labeled food and had regurgitated, they were placed in 1 ml culture solution. Every

Fig. 12.—Loss of ^{35}S -label by *Hydra* on starvation, and increase of ^{35}S -label in the environment over the same period.



twelve hours the *Hydra* were transferred to clean culture solution, and the previous solution was dried and counted for radioactivity. The ^{35}S in the medium was calculated by adding the new radioactivity excreted to the total activity previously excreted. At the end of the experiment the radioactivity of the *Hydra* was counted, and the amount originally present in the animals was calculated. As demonstrated in Fig. 12, the *Hydra* lost about 25 per cent of their total radioactivity to their aqueous environment at a relatively constant rate during five days' starvation. If the ^{35}S is interpreted as representative of *Hydra* protein, then these results suggest that for at least five days the animals were either losing some cellular proteins, catabolizing body protein, or leaking free amino acids. It remains undetermined at present whether this excretion is the result of a general diffusion from all the cells, or by a special excretory mechanism from only those "older" cells near the base.

DISCUSSION

Mechanism of digestion in Hydra.—The results presented in this paper, obtained by chemical and radioautographic methods, are in agreement with the histological evidence contending that most digestion of ingested protein by *Hydra* occurs intracellularly. This conclusion is based on the interpretations of the experiments described in Figs. 2–10; the results all are expected concomitants of intracellular digestion.

The fractionation data (Figs. 2 and 3) support the view that after the food was ingested into the gastrovascular cavity, it was partially degraded into small particles, no free amino acids or peptides being formed. (If significant extracellular digestion had occurred, one might have expected large amounts of amino acids or peptides to accumulate in the gastrovascular cavity.) The particles, as indicated by the Alc-I proteins, were then phagocytized by the gastrodermal cells where they were subsequently digested within food vacuoles.

Further support for the above interpretations is evidenced by the relative absence of significant radioactivity: in the ectoderm ten hours after feeding (Fig. 4); in the regenerated tentacles of *Hydra* decapitated six hours following food ingestion (Fig. 5) as contrasted with *Hydra* decapitated at 48 hours after feeding (Fig. 6); in the lower region of the body tube (Figs. 5, 6, 8, 9, and 10); and, in the ectodermal testes of *Hydra* having a radioautograph taken six hours after feeding. These selective distributions of radioactivity indicate that the food was taken up as undegraded particles into specific regions of the gastroderm, and was not made immediately available to the ectoderm and endodermal cells which were not involved in phagocytosis. This uneven cellular distribution of radioactivity would not be expected in the case of extracellular digestion because the free amino acids would diffuse equally to most cells in this diploblastic tube-like organism.

In addition, the tentacles in radioautographs of *Hydra* fed labeled food showed little activity (Figs. 8–10). However, during digestion it was always observed that fluids and food particles were in the cavity of the tentacles. It is therefore strange that no significant label, which would have been liberated as small diffusible molecules by extracellular digestion, was incorporated into the tentacles.

Still more support for the role played by intracellular digestion is given by the interpretation of the data presented in Fig. 7. In this experiment, amino acids used for the synthesis of hydroxyproline-rich proteins were postulated

to be derived from the gradual breakdown of ingested food within gastrodermal food vacuoles.

Summarizing (1) the data presented in this paper, (2) previous results obtained by following the metabolism of Alc-S proteins [9] (see below) and (3) the histological evidence, all are in agreement concerning the nature of the digestive process carried out by *Hydra*. However, it should be emphasized that any one piece of evidence considered separately is not conclusive in itself and might have an alternative explanation. For example, analysis of the fractionation data still does not eliminate the possibility that free amino acids were released in the gut by extracellular digestion and were immediately taken up by the gastroderm where they were converted to *Hydra* storage protein. However, this possibility seems unlikely because of the preponderance of data pointing to the major role played by intracellular digestion. Thus, upon considering all these results, there appears to be no other means of interpreting the mechanism by which *Hydra* degrades ingested protein.

Comparative aspects of intracellular and extracellular digestion.—It is interesting to consider the results of these experiments in relation to our knowledge of the physiology of digestion. During the process of digestion, relatively large molecules are enzymatically hydrolyzed into simpler compounds. In mammals the food is nearly completely degraded before the products are taken up by the cells lining the gut. Such a degradative process is spoken of as extracellular digestion; it occurs outside of the cells and does not involve the formation of food vacuoles (at least of a size visible by light microscopy). Extracellular digestion usually takes place in relatively large organisms. The products of digestion pass into a fluid transport system which carries the foodstuffs to areas that are too distant to receive the products of digestion by diffusion alone.

In most protozoans and sponges, hydrolysis of foodstuffs occurs only after particulate food is incorporated into an "intracellular gut" or food vacuole. The food vacuole is a cavity separated from the cytoplasmic matrix by a membrane of restricted permeability. Thus, *intracellular* digestion although occurring within a food vacuole, may also be thought of as being extracellular.

The *Hydra* degrades its food by a combination of extracellular and intracellular digestion. This type of mechanism appears to be related in part to the large size of the food ingested as compared to the size of the *Hydra*. Unlike protozoa and sponges, the *Hydra* ingests prey, such as *Daphnia*, which are too large to be phagocytized by the gastroderm. Since the *Hydra* cannot break up the food into small particles by such mechanical means as teeth, gastric mills, etc., it appears that enzymatic [2, 18] and "churning" proce-

dures must exist which might act on the proteins holding the cells together. Thus, these enzymes would act to break apart the food into particles small enough to be engulfed by the gastroderm. At the same time, these enzymes might release a trace of free proteolytic splitting products. (We have recently isolated fluids from the gastrovascular cavity of over one million *Hydra*; this fluid had optimal proteolytic activity at pH 2.5 and 7 [18].) When the particles are of sufficiently small size, they are engulfed by the gastrodermal cells into food vacuoles where the actual hydrolysis of the food into small molecules is carried out. The *Hydra*, being of a simple diploblastic structure, do not need a complex transport system to distribute the hydrolysis products to adjacent cells; the distribution is carried out by diffusion as occurs in sponges. This data provides evidence of a chemical nature for Baldwin's suggestion that "extracellular digestion probably arose in response to the necessity of comminuting relatively large food masses as a preliminary to phagocytosis" [1].

Thus, when speaking of extracellular digestion, we must distinguish between that type carried out by complex animals, such as mammals, and by the type carried out by *Hydra*. In the former, the food macromolecules are normally completely hydrolyzed outside of the cell. In *Hydra*, the food is only partly degraded in the gut into particles to be hydrolyzed further in subsequent intracellular digestion.

The "storage" function of intracellular digestion.—The experiments presented in the present paper indicate that most of the intracellular protein digestion occurring within the *Hydra* takes place over a period of 1–2 days following ingestion. A lag in complete proteolysis is indicated by (1) the retention by the endoderm of most of the radioactive food for one day (Fig. 4), (2) the delayed distribution of radioactivity in the radioautographs of regenerating *Hydra* (Figs. 5 and 6), and (3) the results of experiments described elsewhere [9], demonstrating that Alc-S material serves as precursors for protein at about 1½ days following ingestion (see below). Thus, in intracellular digestion, the length of time required for the conversion of food protein into *Hydra* proteins takes a relatively longer time than in extracellular digestion.

This "storage" of undigested protein by the endoderm may represent more than merely "slow" digestion. It may actually confer some benefits to the animal. In nature, *Hydra* do not always have a continuous supply of food, and one meal may have to last for a day or two. Thus, it would not be efficient for the *Hydra* to break down its food protein immediately and synthesize its own cellular proteins in bursts.

Alcohol-soluble proteins.—The retention by *Hydra* of incompletely digested

food protein for 1–2 days has been observed in experiments previously reported [9, 10]. A striking feature of the earlier work was the unusually high amount of the Alc-S fraction (ca 60 per cent) retained as partial hydrolysis products of the food protein. (Some properties of the Alc-S fraction have been described.) In these experiments [9], the *Hydra* were fed mouse protein unusually high in the radioactive Alc-S fraction (ca 30 per cent of the total radioactivity). During the first six hours after the food was administered to the *Hydra*, the radioactivity in the Alc-S fraction of the animal rose rapidly to over 60 per cent of the total radioactivity and remained at a high level for at least 1½ days. Simultaneously the Alc-I fraction decreased proportionally. There was little change in the amounts of the TCA-S components. The results suggested that during this period half of the Alc-I fraction of the food was partially hydrolyzed intracellularly to the Alc-S material. Most of the radioactive material soluble in alcohol was retained within the *Hydra* cells until 1½ days following ingestion. This experiment reveals another example of the ability of *Hydra* to store food within their cells. By the second day following ingestion, most of the Alc-S fraction was degraded within the cells and was converted to Alc-I cellular proteins [9].

In the present experiments, we were not able to obtain the high yields of the Alc-S material previously reported [9, 10] although a significant increase was observed within the *Hydra*'s cells four hours after ingestion (Fig. 3B). This discrepancy may be due to differences in the preparation and the state of the labeled food proteins. Attempts are now being made to duplicate the previous conditions. In any event, it should be emphasized that the general conclusions derived from both sets of experiments are in accord.

Possible mechanism of egestion.—The results described in Fig. 1, showing the rate at which labeled protein was taken up by the gastroderm, may provide insight into the mechanism by which solid wastes are egested from *Hydra*. Around 3–4 hours following ingestion of food, *Hydra* are observed always to be inflated by fluids to many times their normal diameter. They remain inflated until egestion occurs (usually 5–6 hours after ingestion). The data in Fig. 1 demonstrate that by 4–6 hours after ingestion most of the food was already engulfed by the gastroderm. This rules out the possibility that the fluid contains the “liquefied and hydrolyzed products” of extracellular digestion to be absorbed by the *Hydra*. It appears more likely that the large influx of fluids aids in the subsequent expulsion of undigested solid wastes. The large volume of fluid within the gastrovascular cavity exerts an increased pressure on the circular contractile fibers around the mouth, which expends energy to remain contracted and closed. It is presumed that when the pressure gets too

great, or when the contractile fibers fatigue, the fluid is ejected flushing out the solid wastes.

Applications.—By using the technique described in this paper many new types of investigations become possible. As mentioned under Methods, it is extremely difficult to label *Hydra* by bathing them in solutions of small radioactive molecules. This obstacle to the labeling of *Hydra* can be overcome by incorporating the label into a tissue which the *Hydra* can be stimulated to ingest by the addition of GSH. Similarly, the *Hydra* could be fed labeled live *Daphnia* without added GSH [8]. With labeled *Hydra* available, the techniques of fractionation by differential solubilities, radioautography, separation of cell layers, and grafting unlabeled to labeled *Hydra* should be useful in bringing forth much new information concerning the metabolism and differentiation of this organism.

SUMMARY

1. The mechanism by which *Hydra* degrade ingested labeled protein was investigated, utilizing primarily the techniques of fractionation by differential solubilities and of radioautography.

2. The animals incorporated 80 per cent of the ingested labeled tissue within six hours.

3. No significant increase in amino acids or peptides occurred in the gut during the six hours following ingestion.

4. As the radioactivity of the protein within the gastrovascular cavity decreased, the radioactivity of the protein fraction of the *Hydra* cells increased.

5. At four hours after ingestion, the radioactivity of the large peptide fraction of the *Hydra* cells increased, while a similar decrease was observed in the protein fraction of the *Hydra*.

6. A significant portion of the radioactivity from the ingested tissue was transferred from the highly radioactive endoderm to the ectoderm 24 hours following ingestion.

7. Little of the radioactivity from the ingested food was incorporated into the tentacles regenerating in *Hydra* decapitated six hours following feeding. However, *Hydra* decapitated 48 hours after the labeled food was ingested, had a significant amount of label in the regenerated tentacles.

8. Measurements of a hydroxyproline-rich protein of the nematocysts indicated that synthesis of these proteins occurred for as long as two days following food ingestion.

9. The cellular areas active in taking up the food particles were determined by radioautography.

Digestion of protein in Hydra

10. The animals lost 25 per cent of their ^{35}S -label during the five days following feeding.

11. The following topics are elaborated in the Discussion: mechanism of digestion in *Hydra*; comparative aspects of intracellular and extracellular digestion; the storage function of intracellular digestion; alcohol-soluble proteins; possible mechanisms of egestion; and possible applications of the techniques developed in this study.

I would like to thank Drs. R. Roberts, R. Britten and R. Iverson for their helpful advice. I am especially indebted to Drs. E. Bolton and D. Cowie for much stimulation. Appreciation is extended to Drs. J. F. Woessner, E. L. Chambers and H. A. Schneiderman for their painstaking criticisms of this manuscript.

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C. Mouse

V.C.1 Biochemical and Physiological Differentiation during Morphogenesis, 22, Observations on Amino Acid and Protein Synthesis in the Cerebral Cortex and Liver of the Newborn Mouse¹

(Reprinted by publisher's permission from The Journal of Cellular and Comparative Physiology, vol. 51, no. 3, pp. 385-403, June 1958.)

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The observations reported here were made on the living, newborn mouse. An effort was made to gain estimates of the following quantities in cerebral cortex and liver: (a) the rate at which the carbon of tissue glutamic acid, a non-essential amino acid, is derived from blood glutamic acid; (b) the rate at which the carbon of several non-essential amino acids of the tissues is derived from blood glucose; (c) the rate at which the carbon of certain essential amino acids of the tissues is derived from these amino acids in the blood; and (d) the rate at which the pools of these amino acids in the tissues are drawn upon for protein synthesis. This is the first step in a study of the effect of growth and maturation on these quantities.

METHODS

Glucose uniformly labeled with C¹⁴ (glucose-U-C¹⁴) was prepared from BaC¹⁴O₃, C¹⁴O₂ being synthesized into sugars by *Canna* leaves (Udenfriend and Gibbs, '49; Putnam and

¹ This investigation was aided by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

² We are grateful to Miss Sophie Wysienska for technical assistance.

Hassid, '52). The glucose was repeatedly chromatographed in phenol/water/ammonia (80:20:0.3) until radioactive contaminants (largely fructose) were reduced to less than 1%; phenol was removed from the area of the chromatogram occupied by glucose by running the paper in sec.-butyl alcohol/formic acid/water (70:10:20). With our counting arrangement, using a thin window Geiger tube, the glucose had a specific radioactivity of 3.8×10^4 counts/minute/microgram carbon. Randomly labeled l-amino acids were obtained by growing *Chlorella pyrenoidosa* with $C^{14}O_2$ (+ CO_2) as sole carbon source (Roberts, Cowie, Abelson, Bolton and Britten, '55); after a 10-fold increase in cell mass the protein was separated, hydrolyzed and two-dimensional ascending chromatograms run first in sec.-butyl alcohol/formic acid/water followed by phenol/water/ammonia. After locating the amino acid spots with radioautograms, the spots were cut out, phenol removed by repeated extraction of the paper with equal parts of acetone and ether, the amino acids eluted and then tested for purity by two-dimensional chromatography in the solvents previously used. The amino acids had a specific radioactivity of 2.5×10^4 counts/minute/microgram carbon.

Newborn mice weighing 1.3 to 1.5 gm were injected subcutaneously over the flank with a glass micropipet containing 5.0 microliters of the radioactive material. In experiments lasting longer than two hours animals were returned to the mother shortly after the injection. Adequacy of feeding was checked by observing milk in the stomach through the relatively transparent abdominal wall. At the end of the experiments the animals were placed on ice until spontaneous movements ceased. The chest was then opened, the heart incised and about 60 mg of blood were collected from the thorax in a small amount of heparin. The blood samples were then mixed with 4 ml of ice cold 5% trichloroacetic acid (TCA). Cerebral cortex and liver were promptly removed and 50 mg of each immediately homogenized in 4 ml of 5% ice cold TCA. The subsequent procedure has been fully described (Roberts,

Cowie, Abelson, Bolton and Britten, '55). In brief, after 30 minutes at 5° the homogenate was centrifuged and the supernatant fluid poured off. This is the cold TCA-soluble fraction and is assumed to contain the pool amino acids of the tissues. The precipitate was stirred in 4 ml of 75% ethanol, kept at 40–50° for 30 minutes and then centrifuged. The supernatant fluid was poured off, giving the alcohol-soluble fraction. The precipitate was stirred in 4 ml of solution containing equal parts of ether and 75% ethanol. After 15 minutes at 40–50° the suspension was centrifuged. The supernatant fluid is the alcohol-ether-soluble fraction and was combined with the alcohol-soluble fraction. The precipitate was stirred in 4 ml of 5% TCA and kept in a boiling water bath for 30 minutes and centrifuged. The supernatant fluid is the hot TCA-soluble fraction. The remaining precipitate was washed free of residual TCA, stirred in acidified ethanol and centrifuged; the precipitate was then stirred in ether and centrifuged. The supernatants from these two washes were discarded. The precipitate is the TCA-insoluble or protein fraction. It was demonstrated to be free of glutathione.

In this report we shall confine ourselves to the cold TCA-soluble fraction and the TCA-insoluble fraction. The TCA from the cold TCA-soluble fraction was removed by shaking 5 times each in two volumes of ether and the ether discarded. This material was further fractionated using the cation exchange resin, Dowex 50-X8, in the acid form. In experiments with radioglucose, the fraction containing glucose and lactate which passed through the column was collected and subsequently chromatographed in sec.-butyl alcohol/formic acid/water. Amino acids were eluted from the column with 6 column volumes of 4 N NH_4OH and the ammonia from this fraction driven off on a hot plate. Protein precipitates from cortex and liver were hydrolyzed in 0.3 ml 6N HCl at 106° for 16 hours. Aliquots of the protein hydrolysate corresponding to 20 mg cortex and 10–15 mg liver as well as the total amino acid portion of the cold TCA-soluble fraction were then each dried in a current of air and subsequently taken up in a drop

of H_2O_2 for transfer to the chromatogram paper. Unlabeled protein hydrolysate from liver or cortex and glutamine were added to the chromatograms of the amino acids of the cold TCA-soluble fraction for subsequent ease of identification of amino acids when treated with ninhydrin; γ -amino butyric acid was also added to the fraction from cortex. The fractions were then run on two-dimensional, ascending chromatograms; first in sec.-butyl alcohol/formic acid/water followed by phenol/water/ammonia. Radioautograms were made of the finished chromatograms and the radioactivity of the indicated spots counted directly from the paper. The chromatograms were then treated with ninhydrin for final identification of the spots.

Chromatograms were made of the amino acid portion of the cold TCA-soluble fraction of blood, cortex and liver and of the hydrolyzed protein of cortex and liver. In the experiment with labeled glucose, chromatograms were also made of the glucose-lactate portion of the cold TCA-soluble fraction from blood, cortex and liver. The usual procedure was to count aliquots of fractions in cups and then to determine the proportionate radioactivity of components by counting the spots on the chromatograms; in some instances shortage of material permitted only one of these two procedures. Cup values only are plotted in the graphs to permit comparison among experiments.

Whole blood has been used rather than plasma because of shortage of material and because we have found that for our purposes there is not an important difference in the distribution between plasma and erythrocytes of the tagged amino acids we have used. Distribution ratios have been measured in blood taken from animals after injection of the tracer as well as in samples of blood to which tracer was added *in vitro* (Christensen, Riggs and Ray, '52). Plasma concentrations on occasion were 15% higher than those of cells; in other instances, the two were indistinguishable.

As has been stated, samples of liver and cortex were obtained after bleeding the animal from the heart. The con-

tamination of labeled tissue protein by labeled blood protein does not appear to have been a significant source of error. With labeled glutamic acid the protein of 100 mg blood never contained more than a fraction of a per cent of the radioactivity of the same weight of tissue; with labeled essential amino acids, the protein from 100 mg blood varied from 15% (15 minute sample) to a maximum of 25% (one hour sample) of the radioactivity of 100 mg cortex and from 3% (30 minute sample) to 15% (15 minute sample) of the radioactivity of 100 mg liver. Inspection of the samples of cortex indicated that they contained but little blood.

RESULTS

Injection of l-glutamic acid-U-C¹⁴. This series consisted of 4 littermates which were sacrificed 10, 30, 60 and 240 minutes after subcutaneous injection of uniformly labeled glutamic acid. The first three animals received 1.2×10^5 counts/minute; the last, 10 times this amount. Observations are given in table 1 and figure 1.

One of the aims of these experiments was to estimate the rate at which carbon of blood glutamic acid appears in glutamic acid of cortex and liver. If the blood glutamic acid is an immediate precursor of the glutamic acid of the tissues, that transferred from blood to tissue will have the same specific radioactivity as that in blood. If an intermediate pool such as blood glutamine is present, however, it will dilute the radioactivity of carbon derived from blood glutamic acid and calculations of rates of transfer to tissue based on the assumption that blood glutamic acid is an immediate precursor may be less than the actual rate. Evidence that intermediate pools do not seriously affect rates of transfer calculated on this assumption is provided by finding substantially equal rates at successive periods of time. In such a case, intermediate pools either do not exist to an important degree or their specific radioactivity quickly reaches that of the precursor substance. On this basis and because of the finding that the radioactivity of blood glutamine rises slowly and is

only 17% of the radioactivity in the blood at the end of one hour (table 1), we have considered blood glutamic acid to be an immediate precursor of the glutamic acid of the tissues.

Taking blood glutamic acid carbon as equal to 1.2 $\mu\text{g}/100\text{ mg}$ blood (Albritton, '52), its specific radioactivity was obtained by using the average value of the blood radioactivity-time

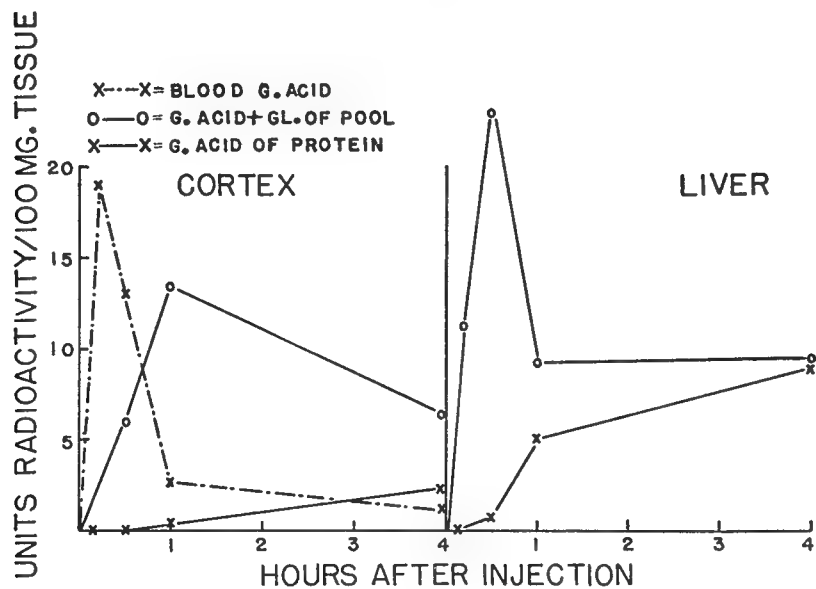


Fig. 1 Radioactivity of glutamic acid (G. Acid) in blood and in protein of cortex and liver and of glutamic acid plus glutamine (Gl) in amino acid pool of cortex and liver, following subcutaneous injection of l -glutamic acid- U-C^{14} . One unit of radioactivity = 64 counts/minute. Since the quantity of tagged glutamic acid in the animal sacrificed at 4 hours was 10 times that used in others of the series, measured values at 4 hours have been correspondingly reduced for this graph.

curve of figure 1 for the desired time interval. Tissue glutamic acid is not a truly stable end product but is degraded and converted to a number of other compounds. At one hour, however, the only known important product is glutamine (table 1). Accordingly we have taken the sum of the radioactivity recovered in glutamic acid plus glutamine of pool and glutamic acid of protein as representing the end products of blood glutamic acid in the tissues.

Using the values of figure 1 at 10 and 30 minutes for the liver and at 30 and 60 minutes for cortex, the following rates were obtained for the transfer of glutamic acid carbon from blood to glutamic acid plus glutamine of liver and cortex, all in microgram C/100 mg tissue/hour: for the liver, 6.0 and 4.2, respectively; for the cortex, 1.1 and 1.6.

A second aim of the experiments was to estimate the rate at which the pool of glutamic acid plus glutamine furnishes amino acid to protein. Since any glutamine which may be present in protein is isolated after acid hydrolysis as glutamic acid, only the sum of the two amino acids in intact protein is known. As an approximation we have consequently considered the pool of glutamic acid plus glutamine as the precursor of glutamic acid recovered from protein. The glutamic acid and glutamine newly incorporated into protein during a given time interval will have the same specific radioactivity as the material of the pool. Accordingly, the rate of incorporation into protein can be expressed in terms of pool size by comparing, during an interval of time, the increment of radioactivity in protein with the average radioactivity of the pool.

Using the increment in tagged carbon present in protein between 30 and 60 minutes (fig. 1), the following values were obtained for the rate at which the combined pool (p) of glutamic acid and glutamine is drawn upon for protein synthesis: for the liver, 0.6 p/hour; for the cortex, 0.1 p/hour, i.e., in the liver 60% and in the cortex 10% of the combined pool of glutamic acid and glutamine appears to be incorporated in protein per hour.

Injection of glucose-U-C¹⁴. This series consisted of 5 littermates which were sacrificed at 17 minutes, 1, 2, 4 and 7 hours after subcutaneous injection of 2.3×10^4 counts/minute of glucose-U-C¹⁴.

The radioactivity of glucose and lactate in the glucose-lactate portion of the cold TCA-soluble fraction was measured after chromatography. Of the total radioactivity in glucose

and lactate, glucose contained the following percentages: for blood, 97–99% except for 88% at two hours; for cortex, 53–56% except for 78% at 7 hours; for liver 90–95% except for 83% at two hours.

With the same approach used for the analysis of observations on glutamic acid, the total amount of tissue amino acid carbon derived from blood glucose/unit time/unit weight of liver or cortex was estimated from the observations of figure 2. Using the values at 17 minutes and one hour (the radioactivity of the amino acids of the pool was added to that of protein) and the value of 100 mg glucose/100 ml blood, which is the average we have found for the newborn mouse, the following rates were obtained for incorporation of blood glucose carbon into carbon of tissue amino acids, all in microgram carbon/100 mg tissue/hour: for the liver, 13 and 14, respectively; for the cerebral cortex, 11 and 19.

We have also been interested in comparing the amount of carbon of an individual amino acid derived by liver or cortex from the amino acid in the blood with the amount of the amino acid synthesized *in situ* from glucose; in this we are presently limited to glutamic acid. It is evident from figure 1 and from the blood level of glutamic acid and glutamine given in figure 2 that the amount of C^{14} derived by either cortex or liver from labeled glutamic acid in the blood in the experiment with labeled glucose is inconsequential. On the basis of the one-hour measurements of figures 3 and 4, the following rates in terms of microgram carbon/100 mg tissue/hour were obtained for incorporation of glucose carbon into carbon of glutamic acid plus glutamine: for the liver, 6.4; for the cerebral cortex, 13. It consequently appears that glucose supplies 10 times more carbon than does glutamic acid of blood to glutamic acid and glutamine of the cortex and an equal amount in the liver.

Finally, again with the same approach used for glutamic acid, the measurements of figures 3 and 4 permit an estimate

of the rate at which the individual amino acid pools (p) are drawn upon for protein synthesis:

AMINO ACID	LIVER/HR.	CORTEX/HR.
Glutamic acid + glutamine	0.8 p	0.1-0.2 p
Alanine	1.5 p	2.2 p
Aspartic acid	4.2 p	0.3 p
Glycine	2.4 p	
Serine	2.8 p	0.7 p

These calculations for the liver have been made from the increments in radioactivity of amino acids of pool and protein between 17 minutes and one hour; subsequent observations yield a substantially smaller value for the apparent rate at which the pools provide amino acids for protein synthesis. By contrast, in the cortex there is good agreement among rates calculated from observations on animals at 1, 2 and 4 hours, e.g., these rates for aspartic acid are 0.3, 0.2 and 0.3 p. It will be noted that this experiment with labeled glucose and that with labeled glutamic acid give substantially the same rates for incorporation of glutamic acid plus glutamine of pool into protein of liver and cortex.

Injection of l-phenylalanine-U-C¹⁴, l-leucine-U-C¹⁴ and l-isoleucine-U-C¹⁴. These three amino acids are not completely separated from one another by the solvents which we have used for chromatography and have consequently been used as a group to study the behavior of essential amino acids. Since the ratio of carbon of phenylalanine to that of leucine and isoleucine is practically the same in the blood of the mouse (Albritton, '52) as in hydrolysate of *Chlorella* protein (Roberts, Cowie, Abelson, Bolton and Britten, '55), it appears that their specific radioactivity in the blood, at least immediately after injection of the labeled amino acids, would be substantially equivalent.

The series consisted of 4 littermates sacrificed at 15, 30, 60 and 240 minutes after subcutaneous injection of the uniformly labeled amino acids. The first three animals received 1.1×10^5 counts/minute; the last, 9.4 times this amount. The observations are given in figure 5 and table 1.

Comparison of figures 1 and 5 show several differences between the behavior of glutamic acid and the three essential amino acids (the figures have been plotted on the basis of equal initial amounts of radioactivity per unit weight animal

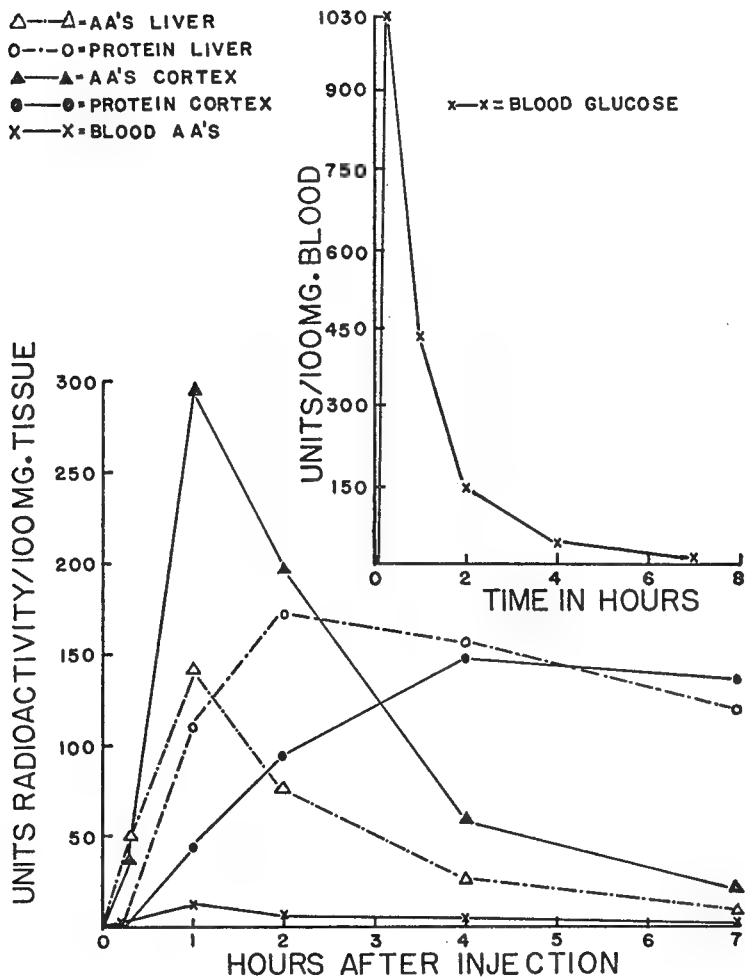


Fig. 2 Radioactivity of glucose (insert) and total amino acids/100 mg blood and of the total amino acids of pool and hydrolyzed protein/100 mg liver or cerebral cortex following subcutaneous injection of glucose- $U-^{14}C$. One unit of radioactivity = 64 counts/minute. A chromatogram of the blood at two hours showed the following amino acids in the indicated percentage of the total radioactivity: aspartic (shadow too low for counting), glutamic acid (18%), serine (25%), glycine (18%), alanine (37%), proline and glutamine (both shadows).

and so are immediately comparable). The blood level of tagged essential amino acids fell more rapidly from its peak than did glutamic acid. Correspondingly, the total tagged amino acid in the tissue (pool + protein) rose more rapidly and to higher levels with the essential amino acids than with glutamic acid. Incorporation of tagged essential amino acids into pro-

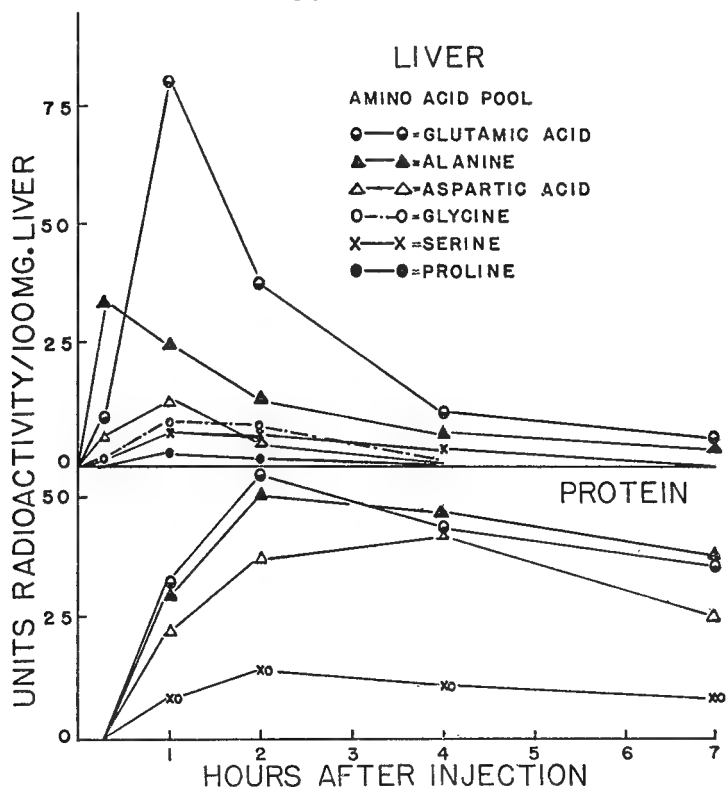


Fig. 3 Radioactivity of amino acids of amino acid pool and of protein hydrolysate of liver following injection of glucose-U- C^{14} . One unit radioactivity = 64 counts/minute. In addition to the amino acids of the figure the following were found to contain C^{14} in the cold TCA-soluble fraction: cysteic acid (shadow at 1 hour; at 2 hours, 1.4; 4 hours, 0.8; 7 hours, 0.8 unit radioactivity); proline (at 1 hour, 0.7; 2 hours, 0.5; 4 hours, 0.2 unit radioactivity and shadow at 7 hours); glutamine (shadow at 17 minutes, 4 and 7 hours; at 1 hour, 0.7; 2 hours, 0.5 unit radioactivity); aspartic acid and glycine (shadows at 4 hours); aspartic acid and serine (shadows at 7 hours). The protein hydrolysate had proline (shadows at 1, 2 and 4 hours; at 7 hours, 0.5 unit radioactivity); arginine (shadows at 1, 2 and 4 hours; at 7 hours, 1 unit radioactivity); cysteic acid (shadows at 4 and 7 hours). Shadows were spots too low for counting.

tein was at a greater rate and reached higher levels than with glutamic acid. No tagged glutamic acid was demonstrable in protein of cortex at 30 minutes and none in protein of liver at 10 minutes; by contrast, there was a substantial amount of

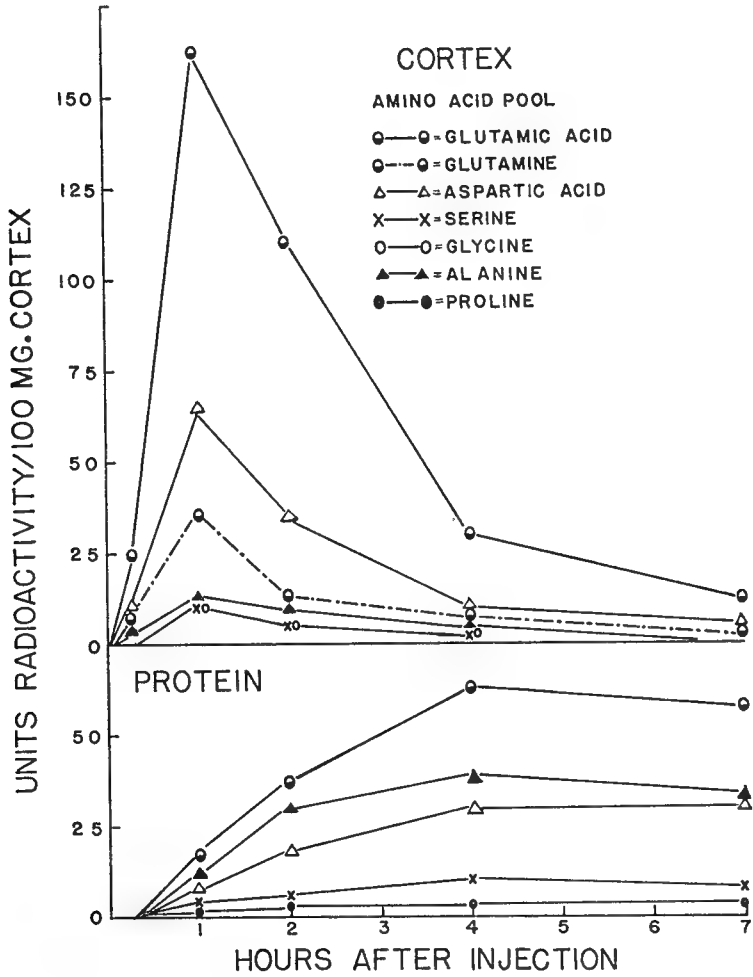


Fig. 4 Radioactivity of amino acids of amino acid pool and protein hydrolysate of cortex following injection of glucose- $U-^{14}C$. One unit radioactivity = 64 counts/minute. In addition to the amino acids of the figure, the following were found to contain C^{14} in the cold TCA-soluble fraction: proline (shadows at 1 and 4 hours; at 2 hours, 1 unit radioactivity); γ -amino butyric acid (shadow at 1 hour; at 2 hours, 6; at 4 hours, 4; at 7 hours, 1 unit radioactivity). The protein hydrolysate also contained glycine (shadows at 1 and 2 hours; at 4 and 7 hours, 1 unit radioactivity) and histidine (shadows at 4 and 7 hours).

tagged essential amino acids in protein of both tissues at 15 minutes. This difference with respect to rate of incorporation into protein was reflected in the behavior of the amino acid pools. In the experiment with tagged glutamic acid, that present in the pool of the cortex was considerably greater

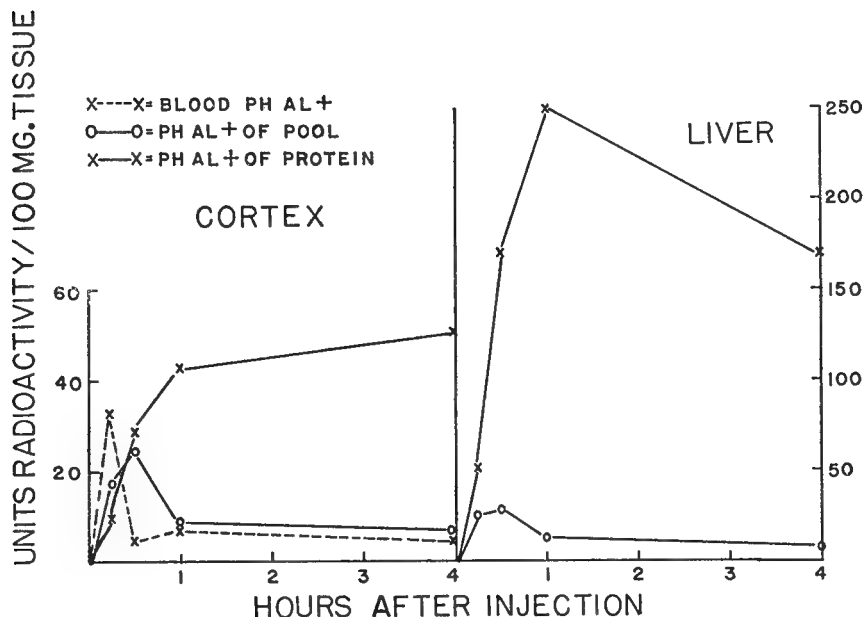


Fig. 5 Radioactivity of phenylalanine, leucine and isoleucine in blood and in amino acid pool and protein of cortex and liver following subcutaneous injection of these amino acids uniformly labeled with C^{14} . One unit of radioactivity = 64 counts/minute. The ordinate on the left is for cortex and blood; that on the right, for liver. Ph. Al. + = phenylalanine + leucine + isoleucine. Measurements at 4 hours have been adjusted for the larger quantity of tagged amino acids injected at this time. To permit direct comparison with the results obtained with glutamic acid (fig. 1), all measurements have been adjusted to the same quantity of radioactivity per unit weight of animal used with glutamic acid.

throughout 4 hours than that in protein; the same held true for liver, at least up to one hour. By contrast, after 30 minutes in the cortex and 15 minutes in the liver, the greater part of the tagged essential amino acids was in protein.

The difference in behavior of the essential amino acids and glutamic acid is further shown by calculations of the kind used

above for glutamic acid. Per unit volume of blood, the total carbon of the three essential amino acids is three times that of glutamic acid (Albritton, '52). To compare rates of transfer of blood amino acid carbon to tissue carbon it is consequently necessary to refer all calculations to a unit weight of carbon of blood amino acid. The results (table 2) show that carbon of the essential amino acids appears 3 to 8 times as rapidly in these amino acids of cortex and liver and 50 to 80 times as rapidly in protein as does that of glutamic acid of blood.

TABLE 2

Rate of transfer of blood carbon in terms of micrograms carbon per 100 mg tissue per hour per microgram carbon of amino acid of blood to total amino acids (pool + protein) and to protein of liver and cortex

The indicated times refer to times after injection of the tracer.
Ph. alanine + = phenylalanine + leucine + isoleucine

AMINO ACID	LIVER TOTAL AA'S		LIVER PROTEIN		CORTEX TOTAL AA'S		CORTEX PROTEIN	
	30' μgm C	60' μgm C	30' μgm C	60' μgm C	30' μgm C	60' μgm C	30' μgm C	60' μgm C
Glutamic acid (1)	3.5			0.5	0.9	1.3		0.05
Ph. alanine + (2)	26	24	23	23	7.0	4.3	4.0	3.8
(2) ÷ (1)	7.5			46	7.8	3.3		76

The high rate of incorporation of the tagged essential amino acids into protein is reflected in the rate at which the amino acid pool is drawn upon for protein synthesis. Using the observations at 15, 30 and 60 minutes, the rate for the liver per hour is 16 p, 17 p and 15 p, respectively; for the cortex, 4.7 p, 4.5 p and 3.6 p, i.e., in the liver an amount of amino acid equal to that present in the pool is incorporated into protein in about 6 minutes; in the cortex in about 15 minutes.

DISCUSSION

The liver and cerebral cortex of the newborn mouse utilize the carbon of glucose for substantial synthesis of non-essential amino acids. A complete, quantitative analysis of the contribution made by all sources to any one of these amino

acids is lacking but the importance of glucose is made evident by the finding that it supplies 10 times more carbon than does blood glutamic acid to glutamic acid and glutamine of the cortex and an equal amount in the liver. Comparable observations with labeled glutamine and other amino acids are necessary to complete the picture. The finding that glutamic acid carbon is derived from phenylalanine, leucine and isoleucine (table 1) indicates its widespread origin.

The difference in rate of incorporation into protein of the tagged essential amino acids and tagged glutamic acid depends in part upon the flow of glucose carbon into glutamic acid and glutamine, and in part upon the difference in concentration of the amino acids in their respective pools. Tagged glutamic acid entering the tissue from the blood is diluted by untagged glutamic acid synthesized *in situ* from glucose. Additional dilution of tagged glutamic acid is caused by the high concentration of this amino acid in the amino acid pool. It has been found in the brain of the adult rat that the molar concentration of glutamic acid is 130X and glutamic acid plus glutamine 200X that of phenylalanine, leucine or isoleucine (Weil-Malherbe, '52). While we have not made quantitative measurements of the pools in the newborn, ninhydrin color values show clearly that glutamic acid and glutamine in both liver and cortex are in very high concentration and the three essential amino acids, in low concentration. Tagged glutamic acid for these two reasons is incorporated slowly into protein and the tagged essential amino acids, rapidly.

The most striking difference in these experiments between the behavior of liver and cortex appears to be in the rate at which the amino acid pools are drawn upon for protein synthesis. With the exception of alanine, all the pools of the liver which we have studied furnish a higher proportion of their amino acid per unit time to protein than in cortex. The greatest difference is with aspartic acid where the turnover rate in the liver is 14 times that in the cortex; with glutamic acid, serine and the essential amino acids, the rate in the liver

exceeds that in the cortex by a factor of about 4. Whether this means a greater rate of incorporation of these amino acids into hepatic protein must wait upon determination of concentrations of amino acids in the pools of cortex and liver of the newborn animal.

Winzler, Moldave, Rafelson and Pearson ('52) have reported on the tagged amino acids found in protein hydrolysates of brain and liver of the newborn mouse after exposure for 24 hours *in vivo* and *in vitro* to glucose-U-C¹⁴. Sky-Peck, Pearson and Visser ('56) have repeated the *in vitro* experiments with brain of the newborn mouse. The *in vivo* results on the liver of the first of these groups of investigators agree with ours with respect to the amino acids in protein which contain C¹⁴ derived from glucose-U-C¹⁴ except that we failed to find evidence of C¹⁴ in methionine and phenylalanine; in cortical protein, unlike their results with whole brain, we failed to find labeling of cystine, lysine, valine, methionine, arginine, leucine and isoleucine but did find C¹⁴ in proline. The low level of radioactivity in all of these amino acids except cystine and the longer exposure to higher levels of glucose-U-C¹⁴ in their experiments may well account for the discrepancies. In our experiments with both tagged glutamic acid and glucose, although certain essential amino acids were found to contain C¹⁴ (in the experiment with tagged glutamic acid, C¹⁴ was present in arginine, phenylalanine, leucine, isoleucine and threonine; with tagged glucose, in arginine and histidine), this was at such a low level that there is no conflict with the viewpoint of the indispensibility of these amino acids both in cortex and liver of the growing mouse. The *in vitro* studies referred to above have shown that under such conditions glucose-U-C¹⁴ contributes C¹⁴ to all amino acids of protein of brain except threonine and has led Winzler and his collaborators to emphasize the caution necessary in this field in transferring findings of *in vitro* experiments to the living animal.

Finally, it may be pointed out that it can be assumed by analogy to the rat (Flexner, '55) that the nerve cells of the

mouse at birth are in the neuroblast stage. The observations that glutamic and aspartic acids are derived from glucose indicates that the citric acid cycle is functional in these immature cells as well as in the liver.

SUMMARY

Using glutamic acid, glucose and a mixture of phenylalanine, leucine and isoleucine, all uniformly labeled with C^{14} , estimates of the following quantities in the cerebral cortex and liver of the living newborn mouse have been made: (a) the rate at which the carbon of tissue glutamic acid and glutamine is derived from glutamic acid of the blood, (b) the rate at which the carbon of several non-essential amino acids of the tissues is derived from blood glucose, (c) the rate at which the carbon of certain essential amino acids of the tissues is derived from these amino acids in the blood, and (d) the rate at which the pools of these amino acids of the tissues are drawn upon for protein synthesis. The importance of glucose carbon for synthesis of glutamic and other non-essential amino acids is pointed out and is related to the low rate of incorporation of labeled glutamic acid in protein as compared to the essential amino acids. The most striking difference in behavior of liver and cortex is in the relatively high rate of turnover in most of the hepatic pools which have been studied.

ADDENDUM

Since the preparation of this paper, pertinent articles on the turnover of proteins of the brain by M. K. Gaitonde and D. Richter (Proc. Roy. Soc. B, *145*: 83, 1956) and A. Lajtha, S. Furst, A. Gerstein and H. Waelsch (J. Neurochem., *1*: 289, 1957) have come to our attention.

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V.C.2 Biochemical and Physiological Differentiation during Morphogenesis, 23, Further Observations Relating to the Synthesis of Amino Acids and Proteins by the Cerebral Cortex and Liver of the Mouse

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THE observations presented here in part supplement previous ones concerning the newborn mouse (FLEXNER, FLEXNER and ROBERTS, 1958). In major part, however, they relate to the adult animal. Both cerebral cortex and liver have been studied to obtain estimates of the following quantities: (a) the rate at which blood supplies glutamic acid to these tissues; (b) the rate at which the carbon of glucose is incorporated into several non-essential amino acids of the tissues; (c) the rate at which certain essential amino acids are supplied to the tissues by the blood; and (d) the rate at which these amino acids in the tissues are incorporated into protein. It has been our interest to study the effect of growth and maturation on these quantities. The findings of GAITONDE and RICHTER (1956) with methionine and of LAJTHA, FURST, GERSTEIN and WAELSCH (1957) and LAJTHA, FURST and WAELSCH (1957) with lysine leave no doubt that amino acids are incorporated at a substantial rate into the proteins of the adult as well as the newborn brain.

METHODS

Glucose randomly labelled with ^{14}C was prepared from $\text{Ba}^{14}\text{CO}_3$, $^{14}\text{CO}_2$ being synthesized into sugars by *Canna* leaves (UDENFRIEND and GIBBS, 1949; PUTNAM and HASSID, 1952). The glucose was repeatedly chromatographed in phenol/water/ammonia (80 : 20 : 0.3 v/v) until radioactive contaminants (largely fructose) were reduced to less than 1 per cent; phenol was removed from the area of the chromatogram occupied by glucose by running the paper in *sec*-butanol/formic acid/water (70 : 10 : 30 v/v). With our counting arrangement, using a thin window Geiger tube, the glucose had a specific radioactivity of 3.8×10^4 counts/min per mg carbon. Randomly labelled L-amino acids were obtained by growing *Chlorella pyrenoidosa* with $^{14}\text{CO}_2$ ($-\text{CO}_2$) as sole carbon source (ROBERTS, COWIE, ABELSON, BOLTON and BRITTEN, 1955); after a tenfold increase in cell mass the protein was separated, hydrolysed and two-dimensional ascending chromatograms run first in *sec*-butanol/formic acid/water followed by phenol/water/ammonia. After locating the amino acid spots with radioautograms, the spots were cut out, phenol removed by repeated extraction of the paper with equal parts of acetone and ether, the amino acids eluted and then tested for purity by two-dimensional chromatography in the solvents previously used. The amino acids had a specific radioactivity of 2.5×10^4 counts/min per μg carbon.

The white mice were young adults, 60-70 days old, weighing 25-30 g. They had free access to food and water. Littermates were used for each experimental group. Equal volumes and, with two exceptions to be noted, equal amounts of radioactive material were injected subcutaneously over the flank in the animals of a group. After an appropriate interval the animals were sacrificed by disloca-

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tion of the cervical vertebrae. The chest was then opened and as much blood as possible was drawn from the heart into a small amount of heparin with needle and syringe. Samples of blood plasma of 0.2 to 0.4 ml obtained by centrifugation were then mixed with 4 ml of ice-cold 5% trichloroacetic acid (TCA). The cerebral cortex and liver were promptly removed and 50 mg of each was immediately homogenized in 4 ml of 5% ice-cold TCA.

The subsequent procedure has been fully discussed (ROBERTS *et al.*, 1955; FLEXNER *et al.*, 1958). In brief, after 30 min at 5° the homogenate was centrifuged and the supernatant fluid poured off. This is the cold TCA-soluble fraction and is assumed to contain the pool amino acids of the tissues. The precipitate was then sequentially treated for 30 min with 75% ethanol, equal parts of ether and 75% ethanol, each at 40–50°, and 5% TCA at 100°. The remaining precipitate was washed free of TCA, stirred in acidified ethanol and centrifuged; the precipitate was then stirred in ether and centrifuged. The precipitate is the TCA-insoluble or protein fraction. It was demonstrated to be free of glutathione.

This report deals with the cold TCA-soluble fraction and the TCA-insoluble fraction. The TCA from the cold TCA-soluble fraction was removed by shaking with 2 vols. of ether and discarding the ether for five successive times. This material was further fractionated using the cation exchange resin, Dowex 50-X8, in the acid form. In experiments with radioglucose, the fraction containing glucose and lactate which passed through the column was collected and subsequently chromatographed in *sec*-butanol/formic acid/water. Amino acids were eluted from the column with 6 vols. of 4 N-NH₄OH and the ammonia was then evaporated.

Protein precipitates from cortex and liver were hydrolysed in 0.3 ml 6 N-HCl at 106° for 16 hr. Samples of the protein hydrolysate corresponding to 20 mg cortex and 10–15 mg liver as well as the total amino acid portion of the cold TCA-soluble fraction were then each dried in a current of air and subsequently taken up in a drop of 30% H₂O₂ for transfer to the chromatogram paper. The fractions were run on two-dimensional, ascending chromatograms; first in *sec*-butanol/formic acid/water followed by phenol/water/ammonia. Radioautograms were made of the finished chromatograms and the radioactivity of the indicated spots counted directly from the paper. The chromatograms were then treated with ninhydrin for final identification of the spots.

Chromatograms were made of the amino acid portion of the cold TCA-soluble fraction of blood plasma, cortex and liver and of the hydrolysed protein of cortex and liver. In the experiment with labelled glucose, chromatograms were also made of the glucose-lactate portion of the cold TCA-soluble fraction from plasma, cortex and liver. The usual procedure was to count samples of fractions in cups (infinite thinness) and then to determine the proportionate radioactivity of components by counting the spots on the chromatograms. Cup values only are plotted in the graphs.

The concentrations of amino acids in the tissue pool and in hydrolysed tissue protein were determined in the following way: known amounts of radioactive amino acid were added to the protein hydrolysate derived from 15 mg liver and 20 mg cortex and, after elution from the column, to the pool amino acids derived from 200 mg tissue. After two-dimensional chromatography the spots were developed with dilute 0.01% ninhydrin, in acetone. Individual spots were then cut from the paper, and eluted with water, NaOH was added to give an alkaline reaction and the solutions taken down to dryness to rid them of ammonia. The residue was next taken up in water, neutralized with HCl and aliquots taken for counting and for determination of amino acids with ninhydrin (ROSEN, 1957).^{*} Blanks were prepared from the chromatographic paper. The ninhydrin value was then corrected for losses in the procedure as indicated by the measured loss in radioactivity. Preliminary experiments with known amounts of amino acids indicated this to be a satisfactory procedure.

Samples of liver and cortex were taken after obtaining as much blood as possible from the heart. As in the newborn mouse, the contamination of labelled tissue protein by labelled blood protein in the adult does not appear to have been a significant source of error. After injection of labelled glutamic acid, the protein of whole blood never contained more than 1 per cent of the radioactivity of protein obtained from the same weight of liver at a corresponding time or 50 per cent of that from the same weight of cortex; after injection of the labelled essential amino acids, these ratios remained less than 5 per cent and 20 per cent. Inspection of the samples of cortex showed that they contained but little blood; this is substantiated by the quantitative observations of LAITHA *et al.* (1957).

^{*} The only satisfactory ninhydrin reagent we have found is that manufactured by Matheson, Coleman and Bell.

RESULTS

Amino acid composition of tissue pools and tissue protein

As shown in Table 1, differences between the composition of the amino acid pool of the newborn and adult liver and cortex do not follow a consistent pattern. In some instances the concentration of the amino acid is greater in the newborn than in the adult (CHRISTENSEN and STREICHER, 1948); in some, the adult values exceed the newborn (WAELSCH, 1951) and in others there is no apparent change with maturation. With respect to the amino acids which we have measured the composition of tissue protein is indistinguishable in newborn and in adult liver or cortex. The values for the amino acid composition of adult liver protein agree with those of SCHWEIGART, GUTHNECK, PRICE, MILLER and MILLER (1949).

Flux of amino acids from blood to tissue

Because of the presumed impermeability of the mature blood-brain barrier to glutamic acid, we have been interested in comparing the rates of transfer of this amino acid from blood to newborn and adult cortex; in both instances a comparison has also been made with rates of transfer of essential amino acids. Phenylalanine, leucine and isoleucine are not completely separated from one another by the solvents we have used for chromatography and have been used as a group to study behaviour of essential amino acids. Since their molar ratio is practically the same in the blood of the mouse (ALBRITTON, 1952) as in hydrolysate of *Chlorella* protein (ROBERTS *et al.*, 1955) their specific radioactivities in the blood after injection are substantially equivalent. In the experiments with labelled glutamic acid, 7.0×10^4 counts/min per g of animal in 0.05 ml were injected subcutaneously; 4.5×10^4 counts/min per g of animal of the essential amino acids were used in 0.05 ml.

The passage of radioactive amino acids from blood plasma to tissue pool to tissue protein in adult mice is shown in Figs. 1 and 2. Similar data obtained with newborn mice have been presented (FLEXNER *et al.*, 1958). Table 2 gives the calculated flux of amino acids from plasma to tissue; the previous data for the newborn mouse are included for comparison.

To calculate the flux from plasma to tissue it is assumed that:

$$F \times \bar{S}_p = \frac{\Delta T^*}{\Delta t}$$

where F is the flux, \bar{S}_p is the average specific radioactivity of the amino acid in the plasma during the interval Δt , and ΔT^* is the increment of the total radioactivity of the amino acid of the tissue. This simplifying assumption, necessary for an amino acid with more than one source, neglects any reverse flow and thereby the flux may be underestimated except at the earliest times.

In calculating the flux of glutamic acid the additional assumption is made that it is the glutamic acid of the blood which supplies radioactivity to the glutamic acid and glutamine found in the tissue. This assumption seems reasonable since the flux is maximal at the earliest observation corresponding to the maximal radioactivity of plasma glutamic acid. In contrast plasma glutamine reaches its maximum at a considerably later time (see Fig. 1). If the radioactivity of plasma glutamine does

TABLE 1.—AMINO ACID COMPOSITION OF TISSUE POOLS AND OF TISSUE PROTEIN

Amino acid	$(\mu\text{g in pool}/100 \text{ mg tissue})$				$(\text{mg}/100 \text{ mg protein})$			
	Liver		Cortex		Liver		Cortex	
	Newborn	Adult	Newborn	Adult	Newborn	Adult	Newborn	Adult
Aspartic	$22 \pm 3.5(5)$	$6.3 \pm 1.0(6)$	$20 \pm 1.6(6)$	$31 \pm 3.0(7)$	$10(1)$	$10(1)$	$11(1)$	$11(1)$
Glutamic	$28 \pm 3.6(5)$	$26 \pm 1.9(6)$	$47 \pm 4.2(6)$	$110 \pm 5.8(7)$	$10(1)$	$9(1)$	$16(1)$	$17(1)$
Glycine	$13 \pm 1.1(6)$	$7.0 \pm 0.7(6)$	$13 \pm 1.1(6)$	$6.3 \pm 0.4(7)$	$4(1)$	$4(1)$	$3(1)$	$3(1)$
Serine	$9.0 \pm 0.8(6)$	$4.8 \pm 0.3(6)$	$7.0 \pm 0.8(6)$	$4.4 \pm 0.4(7)$	$4(1)$	$5(1)$	$4(1)$	$3(1)$
Alanine	$13 \pm 1.2(6)$	$20 \pm 1.7(6)$	$9.0 \pm 1.2(6)$	$7.0 \pm 0.9(7)$	5.1	$6(1)$	$5(1)$	$5(1)$
Ph. al. +	$8.1 \pm 0.9(6)$	$6.0 \pm 0.8(5)$	$6.2 \pm 0.7(6)$	$2.5 \pm 0.3(7)$	$24(1)$	$21(1)$	$14(1)$	$17(1)$
Glutamine	$>10 \pm 1.7(5)$	$>19 \pm 4.0(4)$	$>13 \pm 2.4(4)$	$>22 \pm 4.8(6)$				

Results on the amino acid pools are expressed in terms of the mean and its standard error. Number of samples are in parentheses. Since glutamine is lost on the column (MOORE and STEIN, 1954) the observed values are falsely low. The value for glutamic acid in protein includes glutamine converted to glutamic acid by acid hydrolysis. In this and following tables and graphs ph. al. + = phenylalanine + leucine + isoleucine.

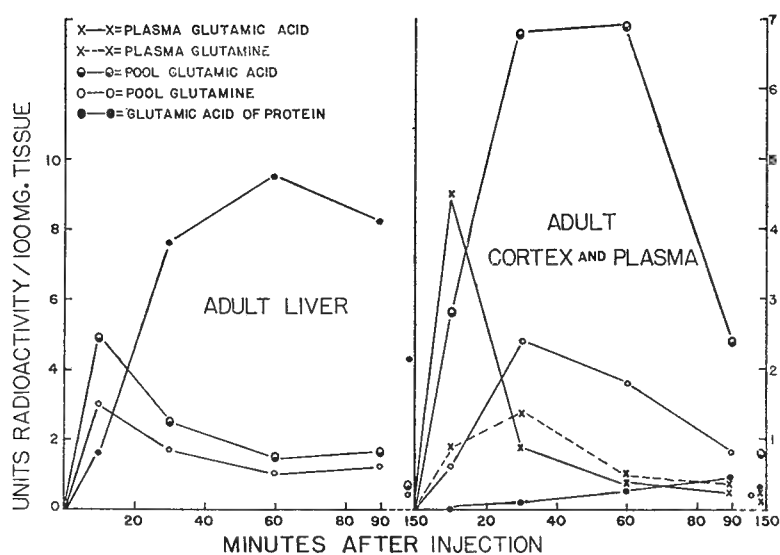


FIG. 1.—Radioactivity of glutamic acid and glutamine in blood plasma and in amino acid pools of cortex and liver, and of glutamic acid in proteins of cortex and liver following subcutaneous injection of *L*-glutamic acid randomly labelled with ^{14}C . In this and following graphs, 1 unit of radioactivity = 64 counts/min. The ordinate on the right is for cortex; that on the left, for liver. At 30 min the protein value of cortex = 0.1 unit radioactivity.

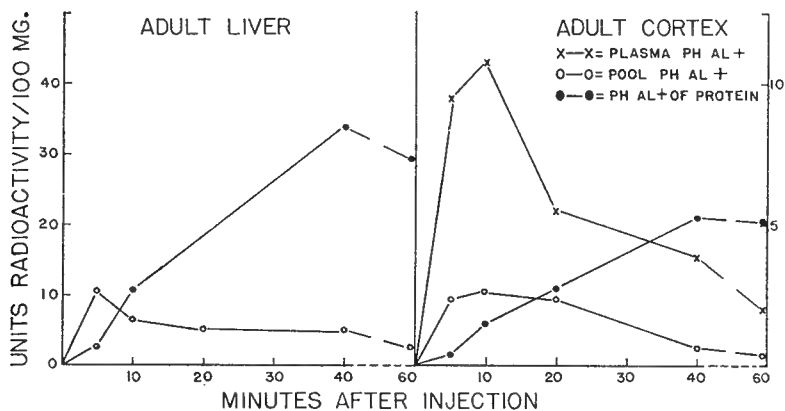


FIG. 2.—Radioactivity of phenylalanine + leucine + isoleucine (Ph. Al. +) in plasma, and in amino acid pools and proteins of cortex and the liver following subcutaneous injection of these amino acids randomly labelled with ^{14}C . The ordinate on the right is for cortex and plasma; that on the left, for liver.

in fact contribute appreciably to the radioactivity of the glutamic acid and glutamine of the tissue, then a correction should be made which would reduce the flux value for glutamic acid. This correction will be relatively small at early times because the ratio of radioactivity of the glutamine to that of glutamic acid is low.

The concentrations of amino acids in the plasma are those given by ALBRITTON (1952). For glutamic acid this is 3.3 mg/100 ml and for phenylalanine plus leucine plus isoleucine, 6.3 mg/100 ml.

TABLE 2.—FLUX OF AMINO ACIDS FROM PLASMA TO TISSUES

Amino acid	Newborn			Adult		
	(μg/100 mg tissue per hr)			(μg/100 mg tissue per hr)		
	t (min)	Cortex	Liver	t (min)	Cortex	Liver
Glutamic	10, 30, 60	—, 3, 4	15, 11, —	10, 30	29, 23	80, —
Ph. al. †	15, 30	28, 23	52, —	5	51	—

The molar concentration in blood plasma of phenylalanine plus leucine plus isoleucine is twice that of glutamic acid (ALBRITTON, 1952); for purposes of comparison half the observed rate of the phenylalanine group has been entered in the table. The times at which the animals were sacrificed after injection (*t*) correspond in order to the values in the following two columns. — indicates data which do not permit calculation of rate at corresponding time.

Synthesis of amino acids from glucose

Following the injection of labelled glucose, we have estimated the total amount of tissue amino acid carbon derived from plasma glucose/unit time per unit weight of tissue and have measured the distribution of this carbon among the labelled amino acids. We have further been interested in comparing the relative importance of plasma glutamic acid and glucose as sources of tissue glutamic acid. Finally, we have been concerned with the possibility that essential amino acids may be labelled with glucose carbon as has been observed *in vivo* in the newborn liver and brain (WINZLER, MOLDAVE, RAFELSON and PEARSON, 1952; FLEXNER *et al.*, 1958).

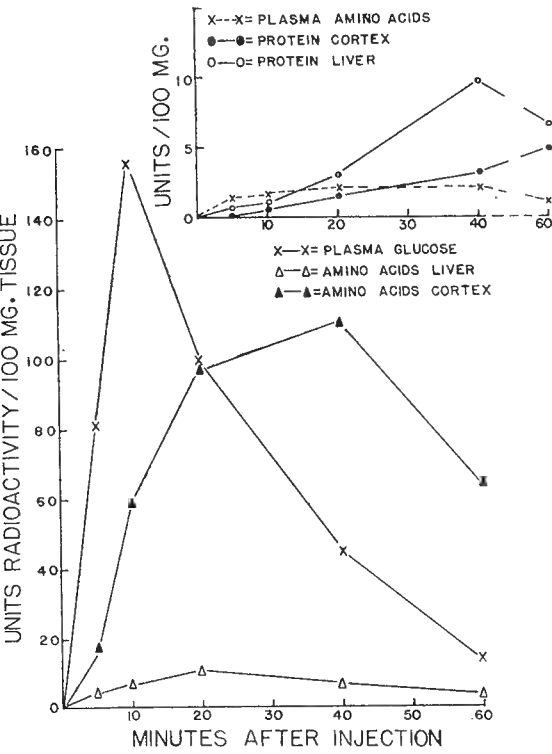


FIG. 3.—Radioactivity of plasma glucose and of total amino acids of pool/100 mg tissue following subcutaneous injection of glucose randomly labelled with ¹⁴C. In the insert, total radioactivity of protein/100 mg tissue and of plasma amino acids/100 mg plasma. Chromatograms of the plasma at 40 and 60 min showed that 8 per cent of the radioactivity of the amino acids was derived from glutamic acid and 8 per cent from glutamine.

FIG. 4.—Radioactivities of individual amino acids of amino acid pool and protein hydrolysate of cortex following injection of glucose randomly labelled with ^{14}C . At 10 min the protein values for aspartic acid = 0.08 unit radioactivity; for alanine = 0.10; for glutamic acid = 0.16. In addition to the amino acids of the figure the following in the cold TCA-soluble fraction were found to contain ^{14}C : γ -amino butyric acid (values midway between aspartic acid and glutamine) and traces of proline; in protein, in amounts too low for counting, were serine, glycine and proline.

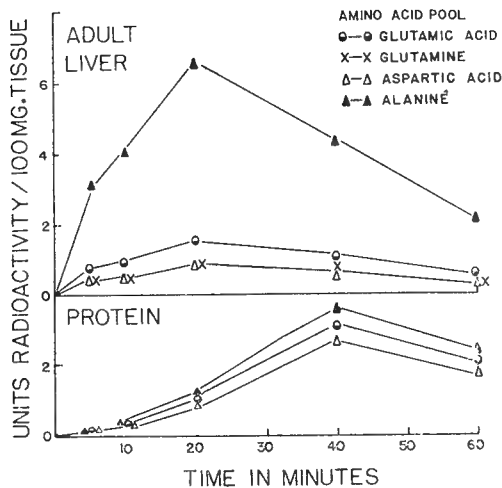
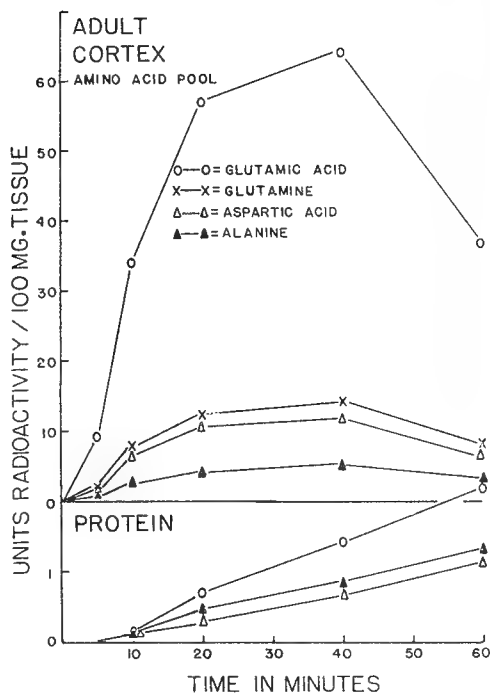


FIG. 5.—Radioactivities of individual amino acids in amino acid pool and of protein hydrolysate of liver following subcutaneous injection of glucose randomly labelled with ^{14}C . At 5 and 10 min, respectively, the protein values for aspartic acid = 0.11 and 0.22; for alanine = 0.15 and 0.30; for glutamic acid = 0.13 and 0.26. In addition to the amino acids of the figure the following were found to contain ^{14}C in amounts too low for counting: in the cold TCA-soluble fraction, cysteic acid, serine and glycine; in protein, serine, glycine and proline.

Animals sacrificed at 5, 10 and 20 min after injection received 9.0×10^4 counts/min per g of animal of labelled glucose in 0.05 ml; animals sacrificed at 40 and 60 min received 1.6×10^6 counts/min per g in 0.05 ml. Values obtained from the tissues of these last two animals were correspondingly reduced for Figs. 3, 4 and 5.

The radioactivity of glucose and lactate in the glucose-lactate portion of the cold TCA-soluble fraction was measured after chromatography. Of the total radioactivity in glucose and lactate, glucose contained the following percentages: for plasma, 80–90 per cent; for cortex, 20–30 per cent; for liver, 89–92 per cent.

The rates of Table 3 have been derived from the observations of Figs. 3, 4 and 5 and from our previously presented observations on the newborn. The calculations

are similar to those described above for amino acid flux. The concentration of glucose in the plasma was found to be 100 mg/100 ml both in the adult and in the newborn mouse. These rates may be minimal since plasma glucose is not the immediate precursor of the labelled amino acids; it is to be noted in the adult, however, that rates obtained soon after injection of labelled glucose are equal to or higher than subsequent ones indicating that intermediate pools quickly reach their maximum specific radioactivities.

TABLE 3.—APPARENT RATE OF INCORPORATION OF GLUCOSE CARBON INTO AMINO ACIDS (TOTAL) AND SPECIFICALLY INTO GLUTAMIC ACID PLUS GLUTAMINE (GLUTAMIC +)

Amino acid	Newborn			Adult		
	(μg C/100 mg tissue per hr)			(μg C/100 mg tissue per hr)		
	<i>t</i> (min)	Cortex	Liver	<i>t</i> (min)	Cortex	Liver
Total	17, 60	11, 19	13, 14	5, 10, 20	210, 190, 120	72, 25, 17
Glutamic +	17, 60	8, 13	3, 6	5, 10, 20	140, 135, 85	16, 5, 3

The times at which the animals were sacrificed (*t*) correspond in order to the values in the following two columns. Late values may be low because reverse flow has been neglected in calculating flux.

In the newborn animal the rates at which glucose supplies carbon for synthesis of non-essential amino acids and specifically for glutamic acid is little or no different in cortex and liver. In the adult cortex the rate at which carbon of glucose is incorporated into non-essential amino acids and specifically into glutamic acid far exceeds that both in the newborn cortex and in the adult liver.

To compare the relative importance of plasma glutamic acid and glucose as sources of glutamic acid in the tissues, the flux values for glutamic acid given in Table 2 must be multiplied by 0.4 to convert them to carbon of glutamic acid. Comparison of these values with those of Table 3 indicate that glucose supplies approximately 10 times as much carbon to glutamic acid of both newborn and adult cortex as does plasma glutamic acid. By contrast, the two sources are of about equal importance in both newborn and adult liver. It is evident from Fig. 1 and from the plasma level of labelled glutamic acid and glutamine given in Fig. 3 that, as in the newborn, the amount of ¹⁴C derived secondarily by either cortex or liver from these amino acids in the blood is inconsequential in the experiment with labelled glucose.

No evidence has been found for labelling of essential amino acids from glucose in the adult animal.

Rates of incorporation of amino acids into protein

The estimated rates at which the amino acids which we have studied are incorporated into protein are given in Table 4. These rates for the newborn have been derived from our data previously presented; those for the adult come from the observations of Figs. 1, 2, 4 and 5. Two points call for comment: first, both radioactive glutamic acid and glutamine are precursors of the glutamic acid isolated from protein after acid hydrolysis. To derive a rate for incorporation of glutamic acid

plus glutamine into protein it is necessary that the specific radioactivities of the two amino acids in the pool be equal. We have not determined the specific radioactivities of the amino acids in individual experiments but have compared the ratios of radioactive glutamic acid to radioactive glutamine in all experiments with the ratio of their mean pool concentrations as given in Table 1. Some glutamine, as has been remarked, is lost on the column but with a constant technical procedure it can be assumed that the loss is relatively constant (MOORE and STEIN, 1954). In practically all experiments except for one series the specific radioactivities of the two amino acids were indistinguishable (Table 1, Figs. 1, 4 and 5). The exception was the cortex of the series of adult mice injected with glutamic acid; in this instance the specific radioactivity of glutamic acid averaged 65 per cent of that of glutamine (Fig. 1).

The second point concerns the use of phenylalanine, leucine and isoleucine as a group rather than individually. This procedure is believed to have led to no important error and had the advantage of placing additional radioactivity at our disposal. As has been said, the molar ratios of these amino acids in the hydrolysate of *Chlorella* protein, used as their source, is practically the same as in the blood of the mouse. Since they are essential amino acids there are no additional sources to distort their specific activities in the pool. We could detect no differences in their specific radioactivities in protein of cortex or liver.

The data of Table 4 lead to the following conclusions:

(1) The apparent rate of incorporation of an amino acid into the protein of the newborn tissues may be greater than, equal to or less than the adult. In the newborn liver the essential amino acids and perhaps aspartic acid are incorporated at a higher rate than in the adult; by contrast, the rate of incorporation of glutamic acid and glutamine in the adult far exceeds that in the newborn; the rates for alanine are indistinguishable at the two stages of development. In the newborn cortex the essential amino acids and alanine are incorporated at a greater rate than in the adult, while aspartic acid, glutamic acid and glutamine appear much the same at the two ages.

(2) The ratio of apparent rates of incorporation of amino acids into protein may differ substantially from the ratio of concentration of these amino acids in the protein. Table 5 gives for each of the amino acids, relative to the phenylalanine group, the concentration ratio in protein and ratio of apparent rate of incorporation into protein. The two ratios for all amino acids agree best in the adult cortex; lack of correspondence is particularly evident in the newborn cortex for aspartic acid, alanine and glutamic acid/glutamine; in the newborn liver for alanine and glutamic acid/glutamine; and in the adult liver for glutamic acid/glutamine.

DISCUSSION

Because of the failure to increase the concentration of glutamic acid in the adult brain by large increases in its concentration in the blood (SCHWERIN, BESSMAN and WAELSCH, 1950; HIMWICH, PETERSEN and ALLEN, 1957), it has generally been assumed that, unlike the immature blood-brain barrier, the mature barrier is impermeable to the amino acid. LAJTHA, quoted by WAELSCH (1958), has shown, however, that labelled glutamic acid in the adult is transferred from blood to brain at rates only slightly less than we have observed in the adult cortex. Indeed our results indicate that flux of glutamic acid from blood to cortex is approximately 10 times greater in

TABLE 4.—FLUX OF AMINO ACIDS FROM POOL TO PROTEIN

Amino acid	<i>t</i> (min)	(μg to protein/100 mg tissue per hr)		<i>t</i> (min)	(μg to protein/100 mg tissue per hr)	
		Cortex	Liver		Cortex	Liver
Ph. al. +	15, 30, 60	27, 23, 24	132, 135, 100	5, 10, 20, 40	11, 12, 10, 11	(35), 55, —, 54
Glutamic + *	10, 30, 60, 240	—, —, 5, 4	—, (4), 15, 10	10, 30, 60, 90	—, 7, 6, 6	110, 116, 100, —
Glutamic + *	17, 60, 120, 240	—, 11, 9, 8	—, 29, 23, —	5, 10, 20, 40, 60	—, 10, 8, 5, 5	156, 104, 120, 160, —
Aspartic*	17, 60, 120, 240	—, 7, 5, 5	—, 86, 55, —	5, 10, 20, 40, 60	—, 7, 5, 4, 4	45, 27, 33, 45, —
Alanine*	17, 60, 120, 240	—, 25, 17, 12	—, 18, 15, —	5, 10, 20, 40, 60	—, 5, 4, 3, 3	24, 14, 17, 22, —
Serine*	17, 60, 120, 240	—, 5, 3, 4	—, 23, 14, —			
Glycine*	17, 60, 120, 240		—, 25, 16, —			

* Starred amino acids are those labelled in the experiments with radioglucose. The times at which the animals were sacrificed after injection (*t*) correspond in order to the values in the following two columns. — indicates data which do not permit calculation of rate of corresponding time. Rates have been calculated from the time preceding the first appearance of measurable amounts of radioactivity in the amino acids of protein, e.g. in Fig. 4 from *t* = 5 min. Glutamine + = glutamine acid plus glutamine.

the adult than in the newborn; and in the adult, on the basis of equimolar concentrations in the blood, is half that of the essential amino acids we have studied.

In spite of the availability of blood glutamic acid to the brain, glucose appears to supply by synthesis *in situ* at least 10 times as much glutamic acid in both newborn and adult cortex as is derived by flux of the amino acid from the blood. That the central nervous system may be unique in this regard is suggested by the contrasting results on the liver which indicate that in this organ the two sources supply approximately equal amounts of glutamic acid.

In vivo experiments on the newborn mouse gave evidence of incorporation of glucose carbon into certain essential amino acids of both brain and liver (WINZLER *et al.*, 1952; FLEXNER *et al.*, 1958). In the adult brain *in vitro* WINZLER and his collaborators found incorporation of glucose carbon into valine and arginine. Our observations made *in vivo* on the adult mouse failed to substantiate these *in vitro* results and, as pointed out by WINZLER and his collaborators, emphasize the caution necessary in this field in transferring findings of *in vitro* experiments to the living animal.

We have used a series of amino acids in an effort to gain insight into the nature of the process of incorporation of amino acids into protein of cortex and liver. Three principal possibilities must be considered: (a) that an amino acid is incorporated into protein solely as a result of synthesis of the complete molecule; (b) that incorporation is the result of exchange reactions involving individual amino acids without

TABLE 5.—CONCENTRATION RATIOS OF AMINO ACIDS IN TISSUE PROTEIN (C.R.) AND RATIOS OF RATES OF INCORPORATION OF THESE AMINO ACIDS INTO TISSUE PROTEIN (R.R.). ALL REFERRED TO PHENYLALANINE, LEUCINE PLUS *iso*LEUCINE

Amino acid	Newborn					Adult				
	Cortex		Liver			Cortex		Liver		
	C.R.	R.R.	C.R.	R.R.		C.R.	R.R.	C.R.	R.R.	
Aspartic*	0.8	0.3, 0.2	0.4	0.7	0.6	0.7	0.6, 0.5	0.5	0.8, 0.7	
Glutamic+	1.1	0.2, 0.2	0.4	0.1, 0.1		1.0	0.6, 0.6	0.4	2.0, 2.0	
Glutamic+*	1.1	0.4, 0.4	0.4	0.2, 0.2		1.0	0.9, 0.6	0.4	3.0, 2.5	
Glycine*			0.2	0.2, 0.2						
Serine*	0.3	0.2, 0.2	0.2	0.2, 0.2						
Alanine*	0.4	0.9, 0.7	0.2	0.1, 0.1		0.3	0.5, 0.4	0.3	0.4, 0.4	

* The first rate ratio is that derived from the first observation after injection of labelled material; the second, is the average of all observations. The two rates in parenthesis in Table 4 have not been used. Starred amino acids are those labelled in the experiments with radioglucose. Glutamic + = glutamic acid plus glutamine.

complete degradation of the molecule, for which evidence can be found in other systems (GALE and FOLKES, 1955; HALVORSON, FRY and SCHWEMMIN, 1955; VAUGHAN and ANFINSEN, 1954; EAGLE, PIEZ and FLEISCHMAN, 1957); or (c) that there is a combination of these two processes. If synthesis were the only process involved, the rate of incorporation of any amino acid should be proportional to its concentration in the protein.

The data of Table 5 show deviations from this rule both in adult and newborn

tissues. These results suggest that exchange may be an important process. They do not provide a complete proof of exchange. It is quite possible that certain proteins of the tissue which are synthesized and degraded rapidly account for most of the incorporation whereas the average composition is determined by other proteins. Exploratory experiments using ion-exchange columns to separate out individual proteins have already shown that different proteins do in fact have different rates of incorporation of amino acids. It seems unlikely, however, that these differences will be sufficient to account for the observations of Table 5.

Additional evidence for exchange comes from the finding that the apparent rate of incorporation of an amino acid in growing tissue may be greater than, equal to or less than in the adult in spite of the close correspondence in the amino acid composition of the protein at the two ages. GAITONDE and RICHTER (1956) have found that the young brain incorporates methionine more rapidly than the old; on the other hand, LAJTHA *et al.* (1957) found no clear difference in the rate of incorporation of lysine into the protein of young and adult liver.

Most of our experiments were carried out for a long enough period to observe the time after injection when the radioactivity of the tissue protein reached a plateau or declined. This time in newborn tissue was invariably longer than in the adult. The observations consequently support the thesis of RITTENBURG, SPROUL and SHEMIN (1948) that during growth there may be a reduced rate of protein breakdown though it is not clear how much of this breakdown is due to true degradation and how much to exchange.

SUMMARY

In contrast to the widely held view that, unlike the immature blood-brain barrier, the mature barrier is impermeable to glutamic acid, the observed flux of glutamic acid from blood to cortex is approximately 10 times greater in the adult than in the newborn. Nevertheless, glucose appears to supply by synthesis *in situ* at least 10 times as much glutamic acid in both newborn and adult cortex as is derived by flux of the amino acid from the blood.

Unlike the newborn, no incorporation of glucose carbon into essential amino acids was observed *in vivo* in the adult cortex or liver.

The apparent rate of incorporation of an amino acid into protein of newborn tissue may be greater than, equal to or less than that found in the adult. Moreover, the concentration ratios of amino acids in tissue protein is not consistently paralleled by the ratios of apparent rates of incorporation of these amino acids into tissue protein. For these reasons it is tentatively suggested that exchange reactions account in part for incorporation of amino acids into protein.

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V.C.3 Lactic Dehydrogenases of the Developing Cerebral Cortex and Liver of the Mouse and Guinea Pig¹

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INTRODUCTION

Experimental evidence based upon several techniques suggests that there are different proteins with common catalytic properties in purified or crystalline preparations of a number of enzymes (Desreux and Herriott, 1939; Paleus and Nielands, 1950; Hirs *et al.*, 1951; Tallan and Stein, 1951). Meister (1950) and Nielands (1952) have shown that Straub's crystalline LDH³ from ox heart muscle

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² We are obliged to Miss Sophie Wysienska for technical assistance.

³ The abbreviations used are: LDH, lactic dehydrogenase; TSM, Tris [tris-(hydroxymethyl)aminomethane], succinic acid, and magnesium acetate in molar concentration ratios of 10:4:5, the stated molarity of the buffer used referring to that of Tris in the mixture; DEAE-cellulose, diethylaminoethyl cellulose; CM-cellulose, carboxymethyl cellulose; APDPN, 3-acetylpyridine analog of DPN (diphosphopyridine nucleotide); Py3AIDPN, pyridine-3-aldehyde analog of DPN.

can be separated into two enzymatically active components. More recent work has added evidence that, among other enzymes, LDH exists in multiple forms with species and tissue specificities (Wieland and Pfeiderer, 1957; Pfeiderer and Jeckel, 1957; Markert and Møller, 1959; Nisselbaum and Bodansky, 1959; Kaplan *et al.*, 1960). In addition, Markert and Møller (1959) have shown that the components of LDH, as separated by electrophoresis, may change during the embryonic development of a tissue. The observations presented here on the cerebral cortex and liver of the mouse and guinea pig are concerned with changes that occur during development in the components of LDH separated by cellulose ion exchangers and starch gel electrophoresis.

METHODS

Mice of the Wistar strain and guinea pigs from our inbred colony were used. Animals were sacrificed by dislocation of the cervical vertebrae. Tissues were quickly excised and, except where noted, immediately transferred to a manually operated glass homogenizer. Homogenates for chromatography were prepared in 5 ml 0.01 M TSM³ pH 7 containing 50 mg deoxycholate. With few exceptions all preparations contained about the same amount of protein. With young animals 600 mg cerebral cortex and 300 mg liver were usually used; with adults these amounts were reduced to 300 mg and 200 mg, respectively. On occasion, material frozen and stored up to a month in a deep freeze at -20°C was used with results indistinguishable from those with fresh tissue.

Two cellulose ion exchangers described by Peterson and Sober (1956) were used. Separation of the anionic components of the homogenates was made on DEAE-cellulose, and the cationic components breaking through were then chromatographed on the cationic exchanger, CM-cellulose. DEAE-cellulose was obtained from Eastman Organic Chemicals. It was prepared by stirring 20 gm into 250 ml 0.1 M TSM pH 7; 0.01 M TSM pH 7 was then added with stirring to give a total volume of 1 liter. The mixture was poured into a liter cylinder and allowed to settle for 30 minutes; the overlaying suspension of fine particles was then siphoned out and discarded. The cellulose was resuspended in 0.01 M TSM, and after settling and separation from the supernatant as before, it was collected and stored at 4°C in a small amount of 0.01 M TSM. CM-cellulose (obtained from the Brown Co., Berlin, New Hampshire) was similarly separated from its fine particles and equilibrated with 0.01 M TSM pH 5.5. The celluloses were packed into columns 20×1.2 cm at a pressure of 4-5 pounds per square inch.

The whole homogenate was placed on the DEAE-column. The column was washed with 40 ml of 0.01 M TSM at a flow rate of 0.5 ml per minute and the wash collected in 20-ml portions. This was done to collect cationic components not adsorbed by DEAE-cellulose.

LDH activity was almost invariably limited to the first 20 ml of wash. Anionic components were then eluted from the DEAE-cellulose using a gradient elution between 50 ml each of 0.01 M TSM and 2 M NaCl in 0.01 M TSM. Eluates of 1 ml were collected at a flow rate of approximately 0.5 ml per minute. The cationic components, which had no affinity for DEAE-cellulose and which were present in 20 ml 0.01 M TSM, were adjusted to pH 5.3 and applied to the CM-cellulose. All LDH activity was retained by the column. The LDH was eluted as with DEAE-cellulose except that a linear gradient was used between 0.01 M TSM and 1 M potassium phosphate both at pH 5.3. All procedures were conducted at room temperature.

LDH activity of the eluates was determined by the colorimetric method of the Sigma Chemical Company. In this method, 0.1 ml of a sample containing LDH is added to 1 ml of a standardized pyruvate substrate containing 1 mg DPNH. After incubation at 37° C for 30 minutes, the enzymatic reaction is stopped by addition of 2,4-dinitrophenylhydrazine which reacts with pyruvic acid to form an intensely colored "hydrazone." The optical density of the solution was measured at 520 m μ in a Coleman, Jr. spectrophotometer. The amount of pyruvate remaining after incubation determined as its "hydrazone" is thus inversely proportional to the LDH activity present in the reaction mixture. The method gives a linear relationship between amount of LDH and optical density over the range of concentration of LDH which we have used. Results are expressed in terms of 1000 Wroblewski units at 25° C (Wroblewski and La Due, 1955). The same method was used for measuring the total LDH activity of a homogenate. Measurements made on an aliquot of the total homogenate and on the supernatant after centrifuging out particulate matter gave the same results.

In addition to chromatography, zone electrophoresis in starch gel (Smithies, 1955) was used to separate the molecular species of LDH. Purified potato starch was obtained from the Mallinckrodt Chemical Works and starch blocks were prepared as described by Markert and Hunter (1959). One part of tissue was homogenized in 5 parts of 0.03 M borate buffer pH 8.5 containing 10 mg deoxycholate per milliliter; the homogenate was centrifuged and the supernatant fluid applied to strips of Whatman No. 1 filter paper. Electrophoresis was carried out at room temperature for 6 hours with a voltage drop of 6 volts/cm. Bands of LDH were visualized with the method of Markert and Møller (1959).

DPN and its analogs, APDPN and Py3AIDPN, were obtained from the Pabst Brewing Company. Rates of reduction of the nucleotides, under the conditions specified in the legend to Fig. 3, were followed at room temperature in the Beckman DU spectrophotometer.

RESULTS

Mouse Tissues

Our results on the cerebral cortex and liver of the mouse have been reported in preliminary form (Flexner *et al.*, 1958-1959).

Figure 1 shows typical elution patterns from DEAE- and CM-cellulose of LDH activity of cerebral cortex of adult and newborn mice. The patterns were highly reproducible both with respect to distribution of amounts of activity among the several peaks and to location of the peaks in the serial fractions of the eluates. Variations in the adult pattern affected the relative heights of peaks A, A', and A'' and on occasion peaks A' and A'' were fused as shown in the newborn cortex. Of these three peaks, A invariably contained more activity than either of the others. A single peak of LDH activity (peak D) was obtained when the break-through from DEAE-cellulose was adsorbed on and eluted from CM-cellulose. The elution pattern of LDH activity of the newborn cortex failed to show components B and C which were invariably present in the adult; components A and D of the newborn occupied the same position as the corresponding components of the adult tissue.

Adult liver lacked all anionic components, i.e., peaks A, A', A'', B, and C, of LDH activity so far as could be judged by elution from DEAE-cellulose. The liver of the newborn and at 6 days of age, however, had a single, relatively weak anionic component corresponding exactly in position to that of peak A of the cortex (Fig. 1) but lacked anionic components A', A'', B, and C. In both adult and young liver, the cationic material not adsorbed by DEAE-cellulose was eluted from CM-cellulose and showed a single peak of LDH activity at the position indicated in Fig. 1 for peak D of cerebral cortex.

Figure 2 gives elution patterns from DEAE-cellulose of proteins of adult cortex and young liver. Comparison of Figs. 1 and 2 shows that the catalytically active components of LDH were well resolved from the bulk of the proteins. The specific activities of peaks A, B, and C of LDH were 100 times that of the initial homogenate. Specific activity is defined as activity of LDH per unit weight of protein.

To test for an analytical or other type of artifact which might account for the finding of apparently multiple components of LDH activity, a series of control experiments was made with DEAE-columns:

Experiment 1. The chromatographic reproducibility of a peak of LDH activity was tested. An adult homogenate was divided into two equal portions. One was placed upon the column and peak A eluted. A known amount of LDH activity from this peak was added to the second aliquot which was then chromatographed. The added activity was recovered quantitatively (better than 90%) in peak A of the second aliquot.

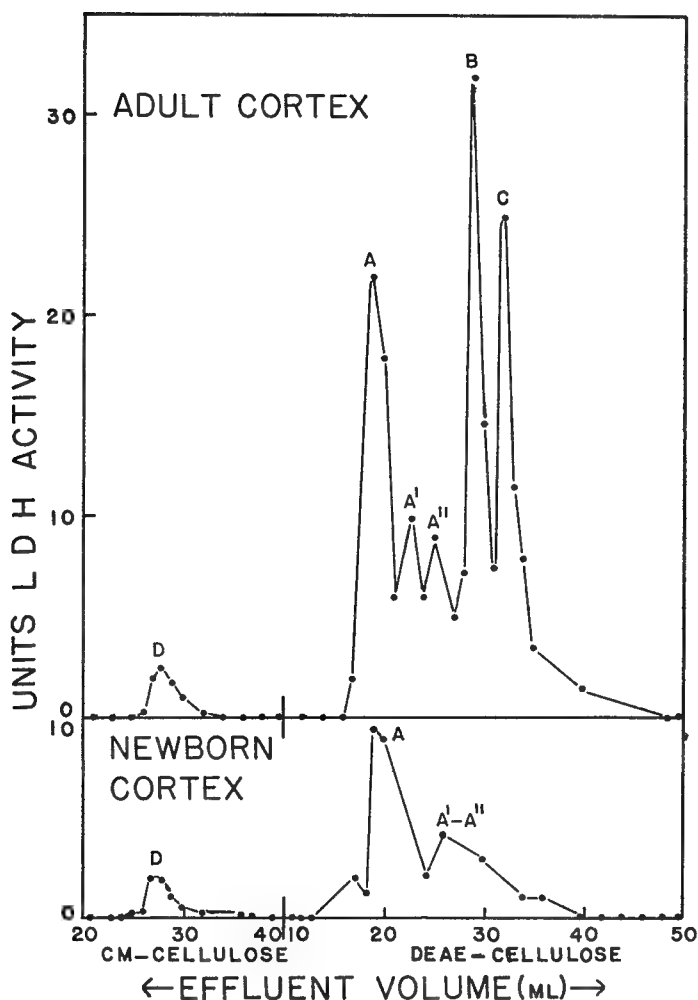


FIG. 1. Chromatographic resolution on CM- and DEAE-cellulose of LDH of cerebral cortex of adult and 12-hour-old mice. One unit = 1000 Wroblewski units at 25° C. Activity is that recovered from 6 mg tissue. Because of variability in loss of activity on the columns, this figure does not give accurate relationships between total anionic and cationic components.

Experiment 2. The possibility was tested that the presence of components B and C in the adult and their absence in the newborn was due to combination of LDH with different components of the two homogenates. LDH from the trailing edge of peak C of adult cortex was added to an homogenate of newborn cortex with subsequent quantitative recovery in its characteristic position after elution from DEAE-cellulose. The next step was to inactivate LDH of a homogenate of adult cortex by placing the homogenate in a water bath at 60° for 10 minutes. This treatment led to loss of 70% of total protein on elution but did not significantly affect the relative amounts of protein along the elution pattern. LDH from peak A of newborn cortex was added to the inactivated homogenate of adult cortex.

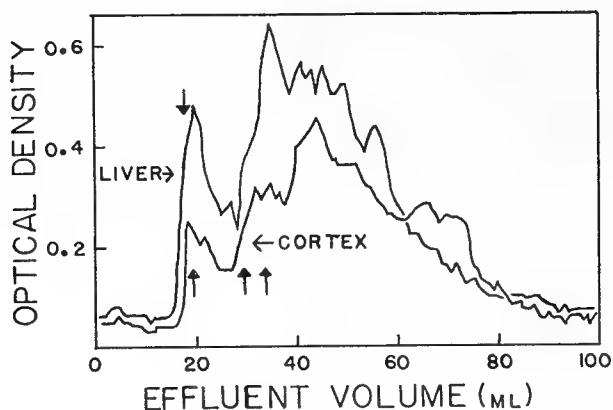


FIG. 2. Elution patterns from DEAE-cellulose of proteins of adult mouse cortex and 6-day-old mouse liver. The location of peaks of LDH activity is indicated by perpendicular arrows. The adult cortex is that of Fig. 1. Protein was measured with the Folin phenol reagent according to Lowry *et al.* (1951). Fractions of eluate were of 1-ml volume. Measurements were made on all fractions to establish the patterns.

Again the added activity was quantitatively recovered in its characteristic position after elution.

Experiment 3. The possibility of postmortem artifact was tested by placing an aliquot of an adult homogenate on the column as soon as possible (within 15 minutes) after death of the animal and comparing the results with those obtained from an equal aliquot kept at room temperature for 2 hours before placing it on the column. The distribution of activity among peaks A + A' + A'', B, C, and D in these two circumstances was indistinguishable.

Experiment 4. The possibility was tested that the additional peaks, B and C, of the adult were due to the action of an enzyme, perhaps, for example a cathepsin or deamidase, present in the adult but absent in the newborn. One part of adult homogenate was added to 8 parts of newborn homogenate and the mixture divided into two equal aliquots. One aliquot was placed immediately on the column and eluted. The other was kept at 37° for 1 hour before placing it on the column. The distribution of LDH activity among the different peaks agreed closely in the two aliquots.

Whole blood of both adult and 6-day-old mice lacked all anionic components and had the same, single cationic peak (peak D) observed with cerebral cortex and liver. Per unit weight, the LDH activity of blood is about 25% of that of liver and about twice that of the cationic component of cortex. In view of the very small amount of blood contained in samples of cortex (Lajtha *et al.*, 1957) and the relatively high LDH activity of the liver, it is evident that the cationic peaks of these two tissues are attributable in major part to the fixed cells of the tissues.

With these experiences from experiments made with the cellulose columns as a background, two additional methods were used in an

effort further to characterize the apparently different molecular species of LDH:

Method 1. Kaplan and associates (1956) have shown that in the presence of lactate the ratios of rates of reduction of DPN and its analogs, APDPN and Py3AIDPN, by crystalline LDH obtained from beef heart differs from ratios obtained with LDH from rabbit skeletal muscle. This approach has been extended to an analysis of the heterogeneity of the LDH's of muscle of numerous species and it has

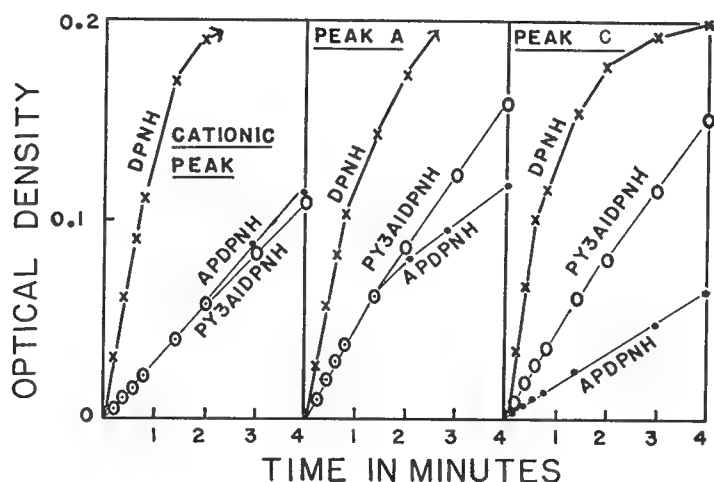


FIG. 3. Rate of reduction of DPN and its analogs with different components of LDH of adult mouse cerebral cortex eluted from DEAE- and CM-cellulose. The reaction mixture contained approximately 0.1 M sodium lactate, 0.1 M phosphate, pH 9, and 200 mg of nucleotide in a total volume of 3 ml. DPNH was measured at 340 $m\mu$; APDPNH, at 365 $m\mu$; Py3AIDPNH, at 355 $m\mu$; all in cuvettes with a light path of 1.0 cm. The rate ratios were not affected by variations in enzyme activity.

been found that the same relative results are obtained with the soluble fraction of a tissue extract as with the purified or crystallized enzyme (Kaplan *et al.*, 1960).

We have used the coenzyme analog approach further to characterize the LDH of the different peaks of adult cortex. Representative reaction rate curves are given in Fig. 3. Peak A gave ratios of the change in optical density with time of DPNH:APDPNH:Py3AIDPNH of 3:1:1; peak C, ratios of 10:1:2.5; and the peak D, ratios of 5:1:1. The initial linear portions of these curves were used to derive these ratios.

The chromatograms suggest the possibility that peak A of adult and newborn cortex and of newborn liver contain a common LDH and that as well the LDH of peak D is identical in the cortex and liver at all ages. In support of this possibility it has been found that peaks which corresponded in the chromatograms of the several tis-

sues gave indistinguishable rate ratios with DPN and its two analogs, i.e., the ratios of peak D of all tissues was 5:1:1; of peak A of adult and newborn cortex and of newborn liver, 3:1:1.

An observation has been made on the cationic component, D, of adult cortex which cannot presently be explained. A comparison was made with all tissues of the rate ratios with DPN and its analogs of the cationic component after adsorption by and elution from CM-cellulose and before adsorption by CM-cellulose, i.e., of the untreated material which broke through DEAE-cellulose. The cationic component of adult and newborn liver and of newborn cortex, obtained in these two ways, gave identical rate ratios. The rate ratios of the cationic component of adult cortex not treated with CM-cellulose consistently differed, however, from those of the component after elution from CM-cellulose and were 2:1:0.5. This finding suggests that one or more of the following possibilities must be considered: (1) that a component of the crude break-through material from DEAE-cellulose altered the rate of reduction of the coenzymes; (2) that cationic LDH from adult cortex was altered on the CM-column; and (3) that in adult cortex there is more than a single cationic component of LDH all but one of which was lost on the CM-column.

Method 2. Starch gel electrophoresis was used to check the separation of apparently different species of LDH from the cerebral cortex obtained with cellulose columns. The results are shown diagrammatically in Fig. 4. With the adult cortex, starch gel electrophoresis, like the columns, showed four major bands of LDH activity, the minor peaks A' and A'' of the DEAE-columns being absent in the starch. With the newborn cortex, electrophoresis, unlike the DEAE-column, showed components B and C though these were present in very small amounts. The intensity of the color reaction used to locate LDH after electrophoresis and the time of the first appearance of color among the several bands indicated that the distribution of LDH activity among the bands did not differ markedly from the distribution of LDH activity among the corresponding LDH fractions separated by cellulose columns.

Starch gel electrophoresis of both adult and newborn liver showed only a single band of LDH activity centered at the approximate location of band D of Fig. 4. This band was very wide, however, overlapping the area occupied by band A of Fig. 4. It is reasonable to suppose that electrophoresis fails to make evident the relatively weak anionic component of LDH activity of young liver, separated by DEAE-cellulose, because of the width of band D.

It will be noted that the behavior of component D as a cation with DEAE-cellulose at pH 7 and its behavior as an anion with electrophoresis at pH 8.5 indicates that it has a relatively high isoelectric point.

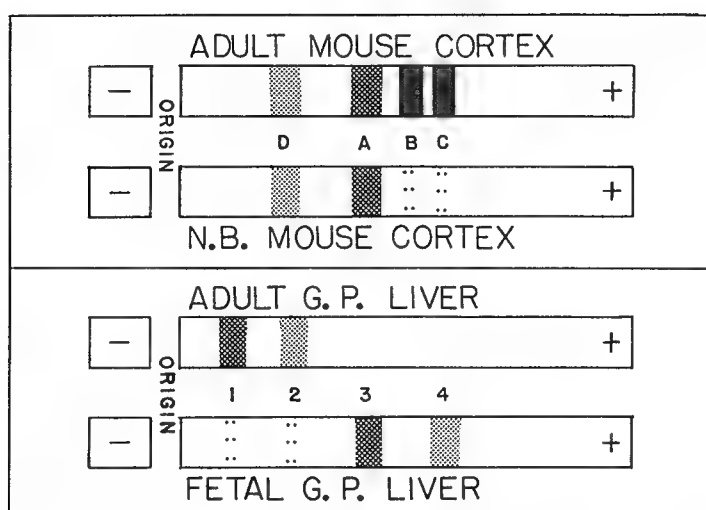


FIG. 4. Bands of LDH activity of adult and newborn (*N. B.*) mouse cerebral cortex and of adult and fetal guinea pig (*G. P.*) liver obtained after electrophoresis. The break in the blocks of the diagrams indicates the place of insertion of the tissue before electrophoresis.

Guinea Pig

In the guinea pig, we have not studied the LDH of cerebral cortex and liver in detail but have been interested only in determining by means of starch gel electrophoresis whether the same type of changes are to be observed during development as occur in the mouse. In this connection, it should be pointed out that the guinea pig is very much more mature at birth than the mouse. The nerve cells of the cerebral cortex of the guinea pig begin to show the characteristics of mature neurons at the forty-first to forty-sixth days of gestation (term 66 days); equivalent changes may be assumed, by analogy to the rat, to occur in the mouse between the eighth and tenth postnatal days (Flexner, 1955). Similarly hematopoiesis in the liver of the rat continues until the twenty-first postnatal day whereas it ceases in the liver of the guinea pig before birth (McKeller, 1949). The cerebral cortex of the adult guinea pig gave an electrophoretic pattern of LDH activity indistinguishable in its major aspects from that of the adult mouse; the cortex of fetal guinea pig at the thirty-third and thirty-seventh days of gestation gave the same electrophoretic pattern as that of the newborn mouse (Fig. 4). In the adult guinea pig, four bands of LDH activity, more sharply delimited than in the mouse, were present in approximately the same position and with the same relative intensity as in the adult mouse. Bands B and C were of about equal intensity, appeared first during color development, and at the end of incubation were much more intense than either A or D. Band A appeared next and was more intense than D. By contrast, the cerebral cortex of the fetal guinea pig had the

greatest LDH activity in band A and the least in bands B and C, which were faintly colored as observed with the newborn mouse cortex.

The results with the liver of fetal and adult guinea pig livers are shown diagrammatically in Fig. 4. As is evident from the figure, the fetal livers (gestation ages 33 and 37 days) had four bands with the greatest LDH activity in band 3 and the least in bands 1 and 2. As in the mouse, the adult liver showed a loss of components. Apparently bands 3 and 4 are lost during development and the major portion of LDH activity in the adult is present in band 1.

Changes in LDH Activity during Development

As shown in Fig. 5, the LDH activity of the cerebral cortex of the mouse is relatively constant up to the twelfth day of life, then rapidly increases approximately threefold to reach the adult level at the end of the nineteenth day. Figure 6 gives an estimate of the extent of participation of the several components of LDH in this increase of

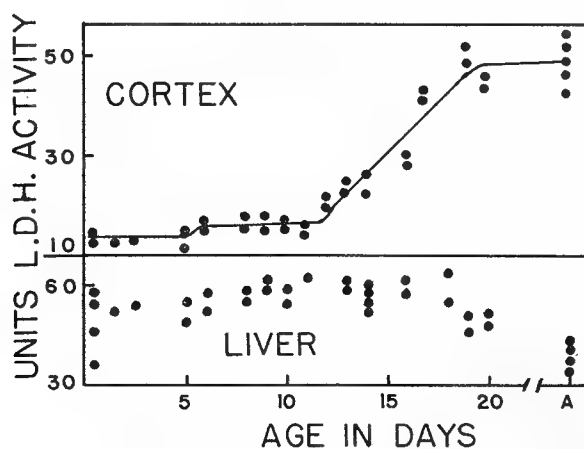


FIG. 5. Total LDH activity of the developing cerebral cortex and liver of the mouse. Units as in Fig. 1. Activity at all ages is that of 6 mg tissue. A = adult.

activity. This estimate was made in the following way. From 40 to 80% (average 60%) of the activity of the homogenate was recovered in the sum of the cationic component which broke through the DEAE-cellulose and the anionic components (A, A', A'', B, and C) eluted from DEAE-cellulose. All the activity of a homogenate of adult liver was recovered in the cationic component after passage through DEAE-cellulose. The original activity of the anionic components in a homogenate of cortex has consequently been estimated on the assumption that it is equal to that of the homogenate minus that of the cationic break-through; i.e., there was no inactivation of the cationic break-through on passage through the DEAE-column. Since the activity of the whole homogenate equals that of the super-

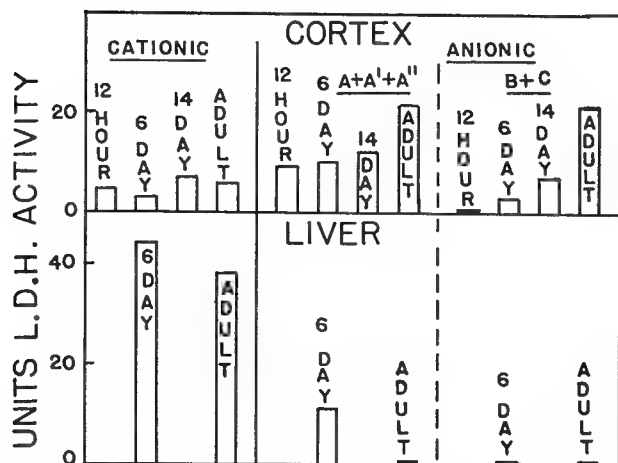


FIG. 6. Changes during development in the activity of components of LDH of cerebral cortex and liver of the mouse. Units as in Fig. 1. Activity of components is that present in 6 mg tissue.

nanant after centrifugation, it is believed that there was no loss of activity because active particulate matter was filtered out by the column. It is further assumed that the loss of activity on elution of the anionic components affected all of them equally. The activity of the cationic component has been taken equal to that of the cationic break-through material before absorption on and elution from CM-cellulose, which results in a 40% loss of activity. On this basis, as shown in Fig. 6, the increase in LDH activity with maturation of the cortex is due to the anionic components with the increment in components B plus C from birth to maturity being approximately twice that of the A components.

As is also shown in Fig. 5, the activity of young mouse liver is about 50% higher than that of the adult. Greater amounts of relatively inactive constituents, such as blood, in the adult may account to an unknown degree for this difference. The greater activity of the 6-day liver, as shown in Fig. 6, appears due in major part to the presence of an anionic component.

DISCUSSION

There are a number of observations to support the view that multiple components of LDH may be present in a tissue. Correlations have been found between the electrophoretic mobility of the components of the LDH of a tissue and their degree of sulfite inhibition (Wieland and Pfeleiderer, 1957). Different components have been found to have distinctive pH optima (Vesell and Bearn, 1958). Crude homogenates of beef heart were resolved electrophoretically into essentially the same components as the crystalline beef heart enzyme (Markert and Møller, 1959). It has been shown here that quantitative differences between the chromatographically separated anionic components of LDH of young and adult cerebral cortex of the mouse

apparently are not due to combination of LDH with different components of the homogenates of the two tissues, to postmortem artifact, or to the action of an enzyme which might modify the LDH molecule and which is more active in the adult than in the newborn. Furthermore, each of the components separated by chromatography when tested had its distinctive activity in the presence of DPN and its analogs. Starch gel electrophoresis confirmed in major part the results obtained with chromatography. The faint presence of components B and C after electrophoresis of newborn cortex and their absence after chromatography appears best explained by the greater sensitivity of the former method. The presence of peaks A' and A'' in the chromatograms is tentatively believed to be because of elution artifact since corresponding components were absent after electrophoresis.

It consequently appears reasonable, as a working hypothesis, to consider the several components of LDH as being distinctive molecular species and as being present in the living cell. A number of enzymes in the developing cerebral cortex of several animals (Flexner, 1955; Kuhlman and Lowry, 1956) have been found to have low levels of activity in the fetal or young animal and to begin to increase rather suddenly early in life to the adult level of activity. The increase in activity of these several enzymes may be caused by activation of mechanisms responsible for synthesis of selected catalytic components such as are represented by components B and C of LDH of the cerebral cortex of the mouse (Fig. 6). This thesis gains some support from our observations on the LDH of the developing cerebral cortex of the guinea pig, which parallel those made on the mouse.

It has been shown that the single component of LDH activity of the adult mouse liver and the two components of the young liver correspond chromatographically to components found in the cerebral cortex. Components of liver and cortex which occupy the same position in the chromatograms have also been found to be indistinguishable in the ratios of their rates of reduction of DPN and its analogs. The demonstration of differences between the characteristics of the total LDH of two tissues, for example by the analog approach, may consequently not necessarily be interpreted to mean that there are no components shared by the two tissues, but may be because several components are present in different proportions.

Satisfactory understanding of the changes which have been found in cortex and liver requires much additional information. If the evidence presented here does in fact mean that multiple components of LDH are normally present in the tissue, how great are the chemical differences among the different components? Are these differences of a basic kind involving variations in amino acid sequences or are they relatively superficial ones related, for example, to changes in the number of amide groups of the same protein molecule? The cerebral

cortex is a complex tissue with several types of glia in addition to its neurons. Are the four LDH's of cerebral cortex to be found in all these cell types or does a distinctive cell type have its distinctive LDH? Finally, in the liver of both mouse and guinea pig, is the loss of one or more components with development to be ascribed to cessation of hematopoiesis and the loss of erythroblasts or to changes within the hepatic cells themselves?

SUMMARY

Using cellulose ion exchangers and starch gel electrophoresis, four components of LDH have been found in the cerebral cortex of mouse and guinea pig. The several components tested could also be distinguished by their ratios of rates of reduction of DPN and two of its analogs. These components apparently each contribute to a different degree to the increase in LDH activity of the cortex which occurs during development.

Maturation of the liver is accompanied by a reduction in the number of components of LDH in both mouse and guinea pig. With the liver of the mouse, peaks of LDH activity in the chromatograms which corresponded in their position with those from cerebral cortex also gave the same ratios of reduction of DPN and its analogs.

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V.C.4 Inhibition of Protein Synthesis in Brain and Learning and Memory following Puromycin*

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ON the assumption that learning and memory may have a biochemical basis, the suggestion has become increasingly frequent during recent years that they may depend in some way on macromolecules such as nucleic acid or protein. The discovery by YARMOLINSKY and DE LA HABA (1960) that puromycin produces profound inhibition of protein synthesis in a cell-free system and the later demonstration that it efficiently suppresses protein synthesis *in vivo* (GORSKI, AIZAWA and MUELLER, 1961) led us to investigate its effect on the central nervous system. This report deals primarily with the substantial suppression of protein synthesis which has been produced in the brains of mice with puromycin, and with the performance of these mice in tests of their ability to learn and to retain memory of the learning experience.

MATERIAL AND METHODS

Biochemical studies

Young adult, albino mice weighing about 30 g were used. We are indebted to Dr. LEON GOLDMAN of the Lederle Laboratories Division of the American Cyanamid Company for our supply of puromycin dihydrochloride pentahydrate. L-Valine uniformly labelled with ^{14}C with a specific radioactivity of 1.0 mc per mg was obtained from the New England Nuclear Corporation.

Subcutaneous injection of puromycin and treatment of tissues. Our first experiments were directed towards establishing the maximum amount of puromycin which could be tolerated in a single, subcutaneous injection and then towards following, as a function of time after injection, the degree of suppression of incorporation of [^{14}C]valine into protein.

Just before use, puromycin dihydrochloride was dissolved in 0.15 ml of water and the solution brought to pH 6 with 1 N-NaOH. This solution was injected subcutaneously over the middle of the back of the animal. At various times after administration of the puromycin, 0.10 ml of [^{14}C]valine (4 μC per 30 g mouse) was injected subcutaneously over the back of the mouse. Forty minutes later the animal was killed by asphyxiation. Previous experience had shown that the rates of incorporation of labelled essential amino acids into the proteins of liver and cerebral cortex are approximately constant during this time interval (ROBERTS, FLEXNER and FLEXNER, 1959). In control experiments, the same amount of [^{14}C]valine was injected in the same way and the animal was killed 40 min later.

In the earliest experiments, samples of cerebral cortex, liver, spleen, kidney (including cortex and medulla) and abdominal muscle were taken for analysis. In later experiments we confined our interests to several regions of the brain. In these last experiments, the brain was removed from the skull and samples were taken of the cerebellar cortex, the two thalami, corpora striata, hippocampi, and the cerebral cortex, the latter divided roughly into rostral and caudal halves. Blunt dissection of the brain to separate these regions required about 3 min. With few exceptions, samples of fresh tissue

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weighing 40–60 mg were used. Pooling of the two thalami, corpora striata and hippocampi yielded about 45 mg of tissue.

Treatment of the tissue followed a modification of the procedure of SIEKEVITZ (1952). Tissue was added to 3.0 ml of ice-cold 6% perchloric acid (PCA) in a glass homogenizer, manually homogenized for 3 min, and kept in ice for 30 min. The suspensions were then centrifuged for 10 min at 3°, the brain samples at 12,000 g, other samples at 1600 g. The supernatant fluid was saved for assay of the radioactivity of free amino acids. To remove all radioactivity except that due to amino acids bound in protein the precipitates were washed and extracted as follows: 3 ml of cold 6% PCA, 3 times; 3 ml of 6% PCA for 15 min at 90°; 3 ml of 4% trichloroacetic acid, 3 times; 3 ml of 95% ethanol for 10 min at 60°; 3 ml ethanol-ether-chloroform (2:1:1, by vol) for 10 min at 50°, twice; 3 ml of ether for 5 min at 20°; and 3 ml of acetone-ether (1:1, v/v) for 5 min at 20°. The final precipitate was suspended in acetone-ether, mixed well and plated with suction on filter paper supported by a steel disk. The weight of this protein was determined to ± 0.02 mg.

Radioactivity was measured with a Baird Atomic gas-flow counter having an ultra-thin window. Corrections were made for sample thickness. Sufficient counts were usually taken to give an error not greater than 5 per cent. Radioactivity of protein was expressed in counts per min per mg; that of the free amino acids, in counts per min per 100 mg wet weight of tissue. Percentage inhibition of rate of incorporation of radioactive valine into protein by puromycin was calculated with respect to the mean of the control series.

In a few experiments measurements were made in control and puromycin-treated animals of the concentration of total free amino acids and of the specific radioactivity of free valine in the liver and cerebral cortex. Total amino acids and the specific radioactivity of valine were measured as previously described (ROBERTS, FLEXNER and FLEXNER, 1959). In brief, total amino acids were determined by the ninhydrin method of ROSEN (1957). To determine the specific radioactivity of valine, the supernatant fluid from 200 mg of centrifuged tissue was passed through the cation exchange resin Dowex 50-8X in the acid form and the amino acids were then eluted from the column with 4 N-NH₄OH. To test for the presence of radioactive substances other than amino acids, the radioactivity of a portion of the eluate from the Dowex column was compared with that of the supernatant fluid before ion exchange. The remainder of the eluate was then run on two dimensional ascending paper chromatograms; first in *sec*-butanol/formic acid/water (70:10:20, by vol) followed by phenol/water/ammonia (80:20:0.3). Radioautograms were made of the finished chromatograms. Individual spots were then cut from the paper, eluted with water and, after removal of ammonia, the radioactivity was measured and valine was determined quantitatively with ninhydrin.

Intraventricular and combined intracerebral and subcutaneous injections of puromycin. Subcutaneous injection of puromycin failed to produce as great an inhibition of incorporation of valine into brain protein as into other tissue proteins (Fig. 1). We therefore directed our attention to achieving increased inhibition by other methods of introducing the antibiotic.

Puromycin was first injected into a lateral ventricle. Intraventricular injections were made with a #27 gauge needle clamped into the rack and pinion of a stereotaxic apparatus. A small piece of polyethylene tubing was placed as a stop over the needle so that it would enter through the skull only to a depth of 2 mm. The mouse was lightly anaesthetized with 2% Evipal (0.01 ml/g), given intraperitoneally. The scalp was then incised and reflected, and a fine hole, just large enough to accept the needle, bored by hand with an awl containing a steel needle. As recommended by HALEY and MCCORMICK (1957) for injections into the posterior horn of the lateral ventricle, this hole was placed 2 mm from the midline on a line running between the rostral points of the base of the ears. Neutralized puromycin in either of two concentrations was mixed with a solution of fluorescein (final concentration, 0.5%) and 0.025 or 0.05 ml of the mixture was slowly injected from a micro-syringe into the ventricle. The scalp was then sewed together. After the desired time interval, radioactive valine was subcutaneously injected and the animal was killed 40 min later. The brain was removed and the spread of the injected material within it was estimated by viewing the distribution of fluorescein under ultraviolet light. Two or three samples of brain were then analysed, comparisons being made of the amount of labelled valine incorporated into protein of regions of the brain showing intense fluorescence and regions in which fluorescence was absent. A control experiment was made with fluorescein alone.

In a second group of experiments an intraventricular injection of puromycin with fluorescein was combined with a subcutaneous injection of puromycin. Comparisons were then made of the amounts of labelled valine incorporated into the protein of fluorescent and non-fluorescent regions of the brain.

In a final series of experiments, six intracerebral injections, all at a depth of 2 mm from the surface of the skull, were combined with a subcutaneous injection. Bilateral injections were made (a) into each of the posterior horns of the lateral ventricles as previously described; (b) through holes in the skull located 1 mm from the midline and 5 mm rostral to the sites of injection into the posterior horn; and (c) through holes in the skull placed on the line between the rostral points of the base of the ears

just above the superior border of the masseter muscle. The six injections were completed in about 10 min and were followed an hour later by a subcutaneous injection of puromycin. The degree of suppression of incorporation of labelled valine into protein was then determined for each of the six areas of the brain already named. In this series the animals were given twice the standard amount of radioactive valine because of the profound suppression of its incorporation into protein.

Behavioural testing

Two procedures were used to assess the effects of puromycin on learning and memory. In one a simple conditioned avoidance response was established in a two-compartment *hurdle box*, divided in half by a 2-in. high hurdle. The left side of the box had a grid floor which could be electrified to shock the animals through the feet and the right side had a smooth white plastic floor. The procedure was to place the mouse in the left compartment, facing away from the hurdle; 5 sec later a 0.5 mA current from a 1000 volt D.C. source was turned on, causing the animal to turn and leap over the hurdle into the 'safe' compartment; 15 sec later, a wire-mesh ladder was lowered into the 'safe' compartment, and the animal was allowed to climb out and given a brief rest-period in a plastic cage; 1 min after the start of the previous trial, the mouse was again placed on the grid in the left compartment and a new trial was begun. Latencies of response were recorded as the amount of time elapsed between placing the mouse on the grid and landing in the 'safe' compartment. Responses of less than 5 sec succeeded in avoiding the shock through the grid and were scored as conditioned avoidance responses.

The second procedure used to test the effects of puromycin on learning and memory was similar to the first but had the advantage of allowing more than one test of learning ability by using the reversal method. This was a discriminative, conditioned avoidance response in a *Y-maze* in which the animal had to learn to avoid shock by running consistently into the left or the right arm of the *Y-maze*. The procedure was to drop the mouse on to the grid floor of the stem of the 'Y' and to turn on the shock after 5 sec. For half the animals, the grid floor of the left arm of the 'Y' was also electrically charged and for the other half, the grid of the right arm was charged. Thus the animals had to learn a right or a left position-habit and a successful response consisted of leaving the stem of the 'Y' within 5 sec and going directly into the correct arm. After entering the correct side, the mouse was allowed to climb up a ladder and was given a rest of about 30 sec before starting the next trial.

In evaluating learning and memory, performance was scored in the majority of animals in terms of the number of trials to reach the criterion of 9 conditioned responses in 10 trials. In an effort to produce a more fragile memory, some animals were trained to a criterion of 3 correct responses in 4 trials. Retention tests were made in one of two ways. Most of the animals were tested by the re-learning method, receiving shock if they failed to perform correctly. A smaller number were tested for retention of the learning experience without shock for the first 10 trials, a test of retention which excluded the possibility of rapid relearning; those animals which showed little or no retention were then retained with shock.

When the hurdle box was used, control and experimental animals were injected subcutaneously with saline or puromycin 1-7 hr before the learning experience and had their retention tests about 24 hr after learning. With the *Y-maze*, animals were trained to give the correct response before injections were made. The next day control and experimental animals were injected subcutaneously with saline or puromycin 2-8 hr before the retention test. Immediately after the retention test, the position habit was reversed and the animals, to avoid shock, were required to go to the opposite side of the *Y-maze*. This procedure provided an assessment of the animal's ability to perform an acquired habit and then to learn a new habit under the influence of puromycin. On the third day tests were given to determine the retention of the more recently acquired, reversed position habit. A few animals were retested for retention of this last habit after an interval of 5 weeks. There were also a few animals which were given puromycin subcutaneously immediately or 24 hr after training in the *Y-maze* and tested for retention 4 days after the injection.

All animals injected subcutaneously with puromycin received 0.42 mg per g mouse. Qualitative observations were made of the behaviour of the mice after puromycin in both the free situation (cage or table-top) and in the testing situation in an effort to note any neurological disturbances the antibiotic might produce. In view of the biochemical results particular attention was given to the appearance of drowsiness or sleep.

RESULTS

Biochemical studies

Tolerance dosage of puromycin subcutaneously injected. In our early experiments, subcutaneous injection of puromycin produced marked suppression of incorporation of labelled valine into the protein of several tissues, with less effect on the brain. We

were primarily interested in obtaining, as far as possible, complete inhibition of incorporation in the brain and consequently needed to know the maximum amount of puromycin compatible with survival of the animal as well as the relative effectiveness of a given amount administered in single or multiple injections. Multiple injections appeared to have no advantage. The maximum single dose which could be tolerated was 0.42 mg per g of mouse and this amount has been used routinely. Occasional animals did not survive this dose beyond 24 or 48 hr but in the great

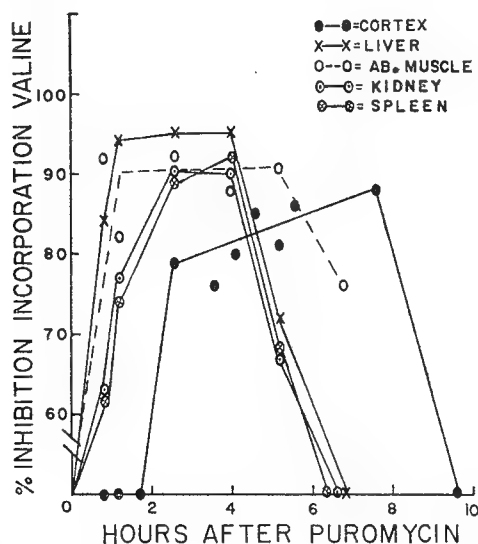


FIG. 1.—Inhibition of incorporation of radioactive valine into protein of mouse tissues at various times after subcutaneous injection of puromycin (0.42 mg per g). A standard amount of valine was injected 40 min before killing the animal. 'Hours after puromycin' refers to the elapsed time between injection of puromycin and death of the animal.

majority of instances it was tolerated and the animals remained in good condition for an indefinitely long period of time.

Suppression of protein synthesis in several tissues by puromycin injected subcutaneously. The degree of inhibition of incorporation of radioactive valine into the protein of liver, spleen, kidney, abdominal muscle, and cerebral cortex is shown (Fig. 1) as a function of time after subcutaneous injection of 0.42 mg of puromycin per gram of mouse. Since we were concerned only with an approximate comparison of the behaviour of these various tissues with that of the cerebral cortex, no effort was made to investigate the degree of variability among a series of animals except with respect to the cerebral cortex. For the other tissues only two control animals were used. Two samples of each tissue from each animal were analysed. The two control values for incorporation of labelled valine into protein differed by 20 per cent for liver, by 4 per cent for spleen; by 25 per cent for kidney; and by 50 per cent for abdominal muscle. The average value was used to determine the degree of inhibition of incorporation of labelled valine in each experiment with puromycin.

In all tissues, except cerebral cortex, maximum inhibition of incorporation of valine into protein was of the order of 90 per cent (Fig. 1). This level was reached or approached within 70 min and was maintained for an additional 3 hr. Cerebral cortex was unique in that inhibition was not apparent within 2 hr of the injection;

after this period 80 per cent inhibition occurred and although it increased somewhat with time, the level was generally below that of the other tissues; it did not decrease until about 8 hr after the injection. It should be noted here, although the point has not yet been thoroughly studied, that recovery from puromycin was accompanied in all tissues by incorporation of a greater amount of radioactive valine than was observed in the control animals.

Suppression of protein synthesis in the brain by puromycin injected subcutaneously. The various areas of the brain in control animals were remarkably alike in the rate at which labelled valine was incorporated into protein (Table 1). The relatively low

TABLE 1.—EFFECT OF PUROMYCIN ON INCORPORATION OF RADIOACTIVE VALINE INTO PROTEIN OF BRAIN

Area of brain	Control	Puromycin-treated	
		Subcutaneous	Intracerebral
	(Counts per min per mg of protein)		
Cerebellar cortex	22	4.0	7.4
Rostral cerebral cortex	25	2.9	2.3
Caudal cerebral cortex	24	2.8	1.3
Corpora striata	23	2.7	1.8
Thalami	18	3.0	1.9
Hippocampi	25	3.3	1.0

The animal given the standard amount of puromycin (0.42 mg per g) subcutaneously received labelled valine 7 hr later and was killed after an additional 40 min. The 'intracerebral' animal received 6 intracerebral injections, each containing 0.13 mg puromycin, followed after 2 hr by the standard subcutaneous injection; labelled valine was given 3 hr afterwards and the animal was killed after an additional 40 min. The 'intracerebral' animal received twice the usual amount of labelled valine; measured radioactivity of protein has consequently been divided by 2 to give the value in the table.

value for the thalamus was not substantiated in the other 6 control experiments of this kind but is included here as an example of the variability which may be observed in a single experiment. The results of one of 5 experiments also show (Table 1) that puromycin injected subcutaneously inhibits incorporation of valine into protein to approximately the same extent in all areas. As noted above, recovery from puromycin in all tissues appeared to be accompanied by an increase of the normal rate of incorporation of valine into protein. In the single experiment made with the different areas of the brain, this apparent increase in rate in all areas was approximately double the highest rate observed in controls.

The six areas of the brain were also studied in a series of 7 control animals. Rostral and caudal cortex alone were studied in an additional 6 animals. The control value for all areas, against which inhibition was measured, was taken as the mean of the value of the cortex of the 13 animals. This appears to be justifiable in view of the close agreement among different areas of the brain and seems preferable because it provides a mean value which rests upon a larger series of animals. This control value for the radioactivity of valine incorporated over the 40-min period was 24 counts per min per mg protein with a standard deviation of ± 5.0 .

The effect of subcutaneously injected puromycin (0.42 mg per g) on the rate of incorporation of radioactive valine was studied in 14 animals. As will be pointed out in the section on behavioural observations, puromycin typically causes drowsiness or

sleep within half an hour after subcutaneous injection and only animals showing these reactions were used for the behavioural studies. Three of the animals used for the biochemical measurements did not show these effects but behaved like wholly normal animals. These three animals were killed 190, 220 and 280 min after puromycin injection and showed, respectively, inhibitions of 16, 54 and 50 per cent. They have not been included in the results shown in Fig. 1 which is limited to the 11 animals which showed the typical behavioural effect of the antibiotic. Results obtained from 7 of these, exposed to puromycin for 2–8 hr, gave a mean value (\pm S.D.) for the radioactivity of valine incorporated into protein of rostral cerebral cortex of 4.1 ± 1.3 counts per min per mg protein. On the average, consequently, puromycin (0.42 mg per g) subcutaneously injected in one dose inhibited incorporation of valine in these animals by 83 per cent. There was some indication that inhibition was greater in the later stages of the inhibitory period.

Suppression of protein synthesis in the brain by intraventricular and combined intracerebral and subcutaneous injections of puromycin. In an effort to obtain greater suppression of protein synthesis, puromycin was injected intraventricularly. A single 0.05 ml injection of a solution of fluorescein containing either 0.26 or 0.52 mg puromycin was made into the posterior horn of a lateral ventricle. The lower dosage of puromycin is sufficient to give an average concentration throughout the brain which is more than five times the concentration maximally effective *in vitro* (YARMOLINSKY and DE LA HABA, 1960). The higher dosage was used in one animal on the chance that it would produce a significant increment of inhibition. After removal of the brain, the spread of the injected material was estimated by viewing the distribution of fluorescence under an ultraviolet lamp. It was assumed that puromycin was present in the areas of brilliant fluorescence. Two of the three animals treated in this way were given radioactive valine an hour after the injection; one animal was given valine 3 hr after the injection; all were killed 40 min later. The animals were in good condition up to the time of death.

Distribution of brilliant fluorescence in these animals essentially followed a common pattern. Brilliant fluorescence was found in all ventricles, in the lateral ventricles principally, in the posterior horns and bodies, and over the caudal portion of both the right and left cortex in a distribution which suggested that staining of the cortex resulted both from escape of fluid to the subarachnoid space from the ventricle along the route of the needle as well as escape from the 4th ventricle. This portion of the cortex appeared to be stained throughout its depth as did grey matter contiguous with the posterior horn. Rostral cortex and rostral periventricular grey matter had no fluorescence.

Intraventricular injections gave an inhibition in stained parts of the brain of 80 per cent, about the same as the average found after subcutaneous injection. Doubling the quantity of puromycin increased inhibition by 6 per cent. The unstained areas of the brain consistently showed an inhibition of 40 per cent indicating that the spread of puromycin from the site of injection was greater than that of fluorescein. Intraventricular injection of fluorescein without puromycin was without effect on the incorporation of labelled valine into protein.

In a second group of two animals, puromycin (0.42 mg/g) was injected subcutaneously and 1 or 4 hr later, 0.26 mg of puromycin in 0.025 ml of water containing fluorescein was injected into the posterior horn of a lateral ventricle. Radioactive valine

was given 1 hr after the intraventricular injection and the animal was killed 40 min later. Both animals were in good condition up to the time of death. Measurements were then made of the radioactivity of the protein of fluorescein-positive and fluorescein-negative areas of the brain. In both instances fluorescein-positive areas incorporated less valine than fluorescein-negative areas. The average effect of supplying puromycin from these two sources in the fluorescein-positive areas was to reduce the rate of incorporation of radioactive valine into the protein to 9 per cent of the normal rate.

A final pair of animals received 6 intracerebral injections of puromycin and, after 2 hours to allow time for spread of the injected material, were given a subcutaneous injection (0.42 mg per g). Preliminary experiments with fluorescein had shown practically complete staining of the brain, except for irregular results with the cerebellum, after the intracerebral injections. Escape of the fluorescein to the subarachnoid space along the route of the needle apparently largely accounted for staining of the cortex. Three hours after the subcutaneous injection of puromycin, labelled valine was given and the animal was killed 40 min later, at which time the animals appeared to be in good condition. The animal which received 6 injections, each containing 0.13 mg of puromycin in 0.0125 ml, showed almost the same degree of inhibition in all brain areas except the cerebellum, the mean inhibition for the remaining 5 areas being 93 per cent. Doubling the amount of puromycin in each intracerebral injection raised this mean value to 95 per cent.

Specific radioactivity of valine of amino acid pools of brain and liver of normal and puromycin-treated animals. The rate at which radioactive valine is incorporated into the protein of a tissue is a function of the specific radioactivity of valine in the amino acid pool. Consequently the inhibition of incorporation of radioactive valine is a direct measure of inhibition of protein synthesis only if the specific radioactivity of the valine pools is essentially the same in control and experimental animals.

For this reason the concentration of 'pool' valine was determined in the cerebral cortex and liver of 2 control mice and of 2 animals after 4-hr exposure to a subcutaneous injection of 0.42 mg of puromycin per g. In addition, the radioactivity of the supernatant fluid of the 6% PCA precipitate was measured in a series of control and experimental animals after it had been demonstrated that only 10 per cent of this radioactivity passed through Dowex 50-8X in the acid form, that all of the measurable radioactivity in the fraction eluted from Dowex was, after chromatography, present in valine and that only traces of radioactivity were present in the aspartic and glutamic areas. In both tissues puromycin was without effect on the concentration of 'pool' valine and it was also without effect on the concentration of total pool amino acids. The mean value for the radioactivity of the 'pool' valine of the cerebral cortex of 7 controls was 179 counts per min per 100 mg tissue with a standard deviation of ± 68 ; the same mean value was obtained for 10 animals exposed to subcutaneously injected puromycin for 1-9 hr but the standard deviation (± 75) was wider. There was no change in the radioactivity of the 'pool' valine of animals receiving intracerebral injections. There was, however, a consistent increase in the radioactivity of 'pool' valine of liver after 4-hr exposure to puromycin. The radioactivity of these livers was 2 to 3 times that of the controls as previously found by NEMETH and DE LA HABA (1962). The values for liver (Fig. 1) were not corrected for the increase of the specific activity of the pool after puromycin; if corrected, the maximum rate of inhibition would amount to 98 per cent rather than 95 per cent as plotted.

Behavioural studies

All animals which had been injected subcutaneously with puromycin (0.42 mg per g) for the behavioural studies became drowsy or sleepy within about half an hour of the injection, recovering gradually to normal wakefulness over the next few hours. All of these animals, many of them drowsy and slow at the time of testing, were responsive to shock and appeared clearly to be activated by the testing situation.

The results of testing for the effects of puromycin, subcutaneously injected, on

TABLE 2.—LEARNING AND RETENTION OF A SIMPLE AVOIDANCE RESPONSE (HURDLE BOX) BY CONTROLS AND BY MICE INJECTED SUBCUTANEOUSLY WITH PUROMYCIN AT VARIOUS TIMES BEFORE THE LEARNING EXPERIENCE (Performance is expressed as the number of trials to reach a criterion of 9/10 correct responses)

Mouse #	Day 1		Day 2	
	Learning experience		Retention	Shock contingency
	Hours after treatment	Trials to criterion	Trials to criterion	
Controls				
1	—	>20	3	Shock in first 10 trials
6	—	11	3	
11	—	10	0	
Puromycin				
7	1	7	2	Shock in first 10 trials
3	1½	16	0	
4	2	17	2	
5	2½	13	0	
10	3½	11	0	
9	4	15	0	
8	5	9	0	
Controls				
2	—	13	7	No shock in first 10 trials;
15	—	15	13	
Puromycin				
12	4½	13	4	shock thereafter
13	5	44	12	
14	7	18	20	

learning and memory were simple and clear-cut. Under all the conditions which were used the mice exhibited normal learning and normal retention. Results of tests in the hurdle-box (Table 2), show that the control group (5 animals) and the experimental group (10 animals) which received puromycin 1 to 7 hours before the learning trials needed essentially the same experience to reach the criterion of 9 conditioned responses in 10 trials. Only mouse #13 of the puromycin group showed delayed learning. Tests made the next day, whether by the relearning method with shock, or without shock for the first 10 trials, showed that the antibiotic had not affected retention of the conditioned response. Results (Table 3) substantiating this conclusion were obtained with the Y-maze. In this experimental situation the animals learned on day 1 to a criterion of either 9 conditioned responses in 10 trials or 3 conditioned responses in 4

trials (fragile memory). On day 2 the controls (4 animals) were injected subcutaneously with saline and the experimental group (10 animals) with puromycin 2–8 hr before the retention tests. On retention testing the groups showed equally good retention of the learning experience of the previous day. Reversal learning was instituted immediately after the retention test and again the puromycin group performed as well as the controls. On day-3 no distinction could be made between the 2 groups in their retention

TABLE 3.—LEARNING AND RETENTION OF A DISCRIMINATION AVOIDANCE RESPONSE AND ITS REVERSAL (Y-MAZE)

Mouse # Y-series	Day 1	Day 2		Day 3	
	Learning Trials to 9/10	Retention test		Reversal learning Trials to 9/10	Retention of reversal Trials to 4/5
		Hours after treatment	Trials to 4/5		
Saline Control					
1	>40	2½	0	29	8
2	25	5	7	20	0
Puromycin					
3	19	2	0	10	0
4	21	3	2	15	0
5	23	5	3	13	0
6	6	6	0	9	0
41	6	7	0	4	0
42	9	8	0	11	0
Trial to 3/4					
		Saline Control	Trials to 3/4	Trials to 3/4	Trials to 4/5
10	9	2	10	6	5
13	17	5	7	>40	12
Puromycin					
8	13	2½	21	4	12
12	5	3	2	15	0
9	13	5	9	8	5
11	20	5¼	12	12	0

Retention tests of the learning experience of Day 1 were made on Day 2 at the indicated time after subcutaneous injection of saline or puromycin. Reversal learning was started immediately after the retention tests. Retention tests for the reversal learnings were made on Day 3. Performance is expressed as the number of trials to reach the indicated criteria. Both retention tests were made by the relearning method.

of reversal learning. As shown in Table 4, subcutaneous injection of puromycin immediately after the learning experience in the Y-maze, or 24 hr later, was without effect on the retention of the learned experience when this was tested 4 days after treatment with the antibiotic. Finally, good retention of reversal learning was demonstrated in a few control and puromycin-treated animals 5 weeks after the learning experience.

Efforts have also been made to test learning and retention in animals which received intracerebral injections of puromycin. At the present time it is premature to report on these observations except to remark that intracerebral injections in the amounts used

for the biochemical studies presented here produced a degree of disorientation incompatible with learning or memory. Results of some promise have, however, been obtained with reduced amounts of puromycin injected intracerebrally; this aspect of the problem is presently being pursued.

TABLE 4.—LEARNING AND RETENTION OF A DISCRIMINATION AVOIDANCE RESPONSE (Y-MAZE)

Mouse # Y-series	Day 1 Learning Trials to 9/10	Treatment (Hours after learning)	Day 4 or 5 Retention Trials to 9/10
<i>Saline</i>			
38	14	0	2
36	13	24	3
<i>Puromycin</i>			
40	8	0	0
35	3	24	0
37	3	24	2

Subcutaneous injections of saline or puromycin were made either immediately or 24 hr after learning. Retention was tested by the re-learning method 4 days after the injections. Performance is expressed as the number of trials to reach a criterion of 9 out of 10 correct responses.

DISCUSSION

In the experiments reported here, learning and tests for its retention have been scheduled in light of the finding that protein synthesis in the brain is substantially suppressed during the interval from 2 to 8 hours after subcutaneous injection of puromycin (Fig. 1). The learning experiences and retention tests in both the hurdle-box and Y-maze were planned with two ends in view: (1) to test for learning ability after protein synthesis had been suppressed for various periods of time; (2) to test for retention when the puromycin effect was present for various periods of time in the post-learning period. The experiments of Tables 2 and 3 provided for an experience in learning or reversal learning extending from the beginning of the period of suppression of protein synthesis after an injection (injection 2 hr before learning) to the end of this period (injection 8 hr before learning). These experiments, supplemented by that in which an injection was made immediately after the learning experience (Table 4), provided for tests of retention of learning or reversal learning of animals in which protein synthesis had been suppressed for periods up to 8 hr after the learning experience. Retention tests were also made of animals in which protein synthesis was suppressed for periods of 6 hr or less preceding the tests (Table 3, injection 8 hr before retention test) and of animals in which synthesis was suppressed 24 hr after the learning experience and 4 days before retention testing (Table 4).

Failure to observe any interference with learning or its retention in these experiments is not to be taken as conclusive evidence that learning and memory are independent of protein synthesis, although the biochemical evidence indicates that on the average puromycin suppressed protein synthesis by 83 per cent in all areas of the brain which we have studied. This effect may be far from uniform on individual cells or individual proteins. Indeed the residual protein synthesis which we have not been able to inhibit by subcutaneous injection of puromycin may be sufficient to provide,

in our testing situation, for learning and its retention. Some of these doubts can perhaps be resolved by using column chromatography for study of the puromycin effect on different proteins and autoradiography for an analysis of the degree to which protein synthesis is suppressed among different cells. It is to be hoped, however, that a way will be found to suppress protein synthesis more completely without damaging unduly the behavioural status of the animal as occurred in experiments reported here when subcutaneous injections were combined with intracerebral injections.

SUMMARY

Subcutaneous injections of the antibiotic, puromycin, have been found to suppress incorporation of radioactive valine into the protein of several areas of the mouse brain by 83 per cent on an average. This apparent degree of inhibition of protein synthesis, maintained for various periods of time, was without effect on the learning and retention of simple or discrimination avoidance responses.

Inhibition of incorporation of valine into protein was raised to 95 per cent by combining subcutaneous and intracerebral injections of puromycin. Animals treated in this way, however, showed disorientation incompatible with learning or memory.

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V.C.5 Memory in Mice as Affected by Intracerebral Puromycin

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Abstract. The antibiotic, puromycin, caused loss of memory of avoidance discrimination learning in mice when injected intracerebrally. Bilateral injections of puromycin involving the hippocampi and adjacent temporal cortices caused loss of short-term memory; consistent loss of longer-term memory required injections involving, in addition, most of the remaining cortices. Spread of the effective memory trace from the temporal-hippocampal areas to wide areas of the cortices appears to require 3 to 6 days, depending upon the individual animal. Recent reversal learning was lost while longer-term initial learning was retained after bilateral injections into the hippocampal-temporal areas.

The suggestion has become increasingly frequent during recent years that nucleic acids or proteins may be concerned with learning and memory. We were led to investigate the effects of the antibiotic, puromycin, on these aspects of behavior by the discovery of Yarmolinsky and de la Haba (1) that puromycin produces profound inhibition of protein synthesis in a cell-free system and by the later demonstration that it efficiently suppresses protein synthesis in vivo (2). In an earlier paper with de la Haba and Roberts (3) we have reported studies on mice which received the maximum amount of puromycin which could be tolerated in a single subcutaneous injection. Although this treatment appeared to suppress the rate of protein synthesis in various parts of the brain to 80 percent of the control value for a period of 6 hours, it was without effect on the learning and retention of simple or discrimination avoidance responses.

The experiments to be reported here have been made with intracerebral injections of puromycin. The amounts injected were smaller than previously

used (3) so that disorientation of the animal at the time of testing was avoided. With this intracerebral approach, we have found that memory can be consistently destroyed, difference in the effective loci of recent and longer-term memory apparently established, and the time factor concerned in modification of the effective locus determined. Upon recovery, animals were capable of learning again. We cannot now relate these behavioral effects to suppression of protein synthesis, since our biochemical studies are not yet complete.

Adult white mice were trained in a Y-maze with a grid floor through which shock could be applied. The animal was placed in the stem of the Y. To avoid shock the mouse had to move into the correct arm within 5 seconds. If it entered the incorrect arm, it received shock until it moved to the correct arm. Training was continued in one session of about 20 minutes to a criterion of 9 out of 10 correct responses, thus avoiding overtraining. The same procedure was used in testing for memory of the training experience,

shock having been given for errors of performance except as noted. In this type of training we have found mice to behave essentially like rats and to retain excellent memory of the training for at least 5 weeks. Intracerebral injections of puromycin, each injection of a volume of 0.012 ml, were made through small holes in the skull, as previously described (3).

From one to three injections of puromycin were made into each hemisphere, all at a depth of 2 mm from the surface of the skull. Bilateral injections were made through holes placed (i) just above the angle between the caudal sutures of the parietal bones and the origins of the temporal muscles—these are here designated temporal injections; (ii) 2 mm lateral to the sagittal suture and 2 mm rostral to the caudal sutures of the parietal bones—these are here designated ventricular injections; and (iii) 4 mm rostral to these last holes and 1 mm lateral to the sagittal suture—these are here designated frontal injections. At the present time we are dependent upon control injections of a solution of fluorescein (3) to estimate the spread of puromycin; more refined studies, using other techniques, are not yet complete. Animals which receive injections of fluorescein were sacrificed 1 hour after the injection.

Results with four of the seven types of injections are shown in Fig. 1. This shows that the area around the caudal rhinal fissure was stained with temporal but spared with ventricular injections. The three types of injection not shown in the figure consisted of combined ventricular and temporal injections, of combined ventricular and frontal injections, and of combined temporal and frontal injections. The distribution of fluorescence in these combined injections was essentially the sum of the individual injections as shown in Fig. 1.

The effects of intracerebral injections of puromycin on memory of the training experience are given in Table 1,

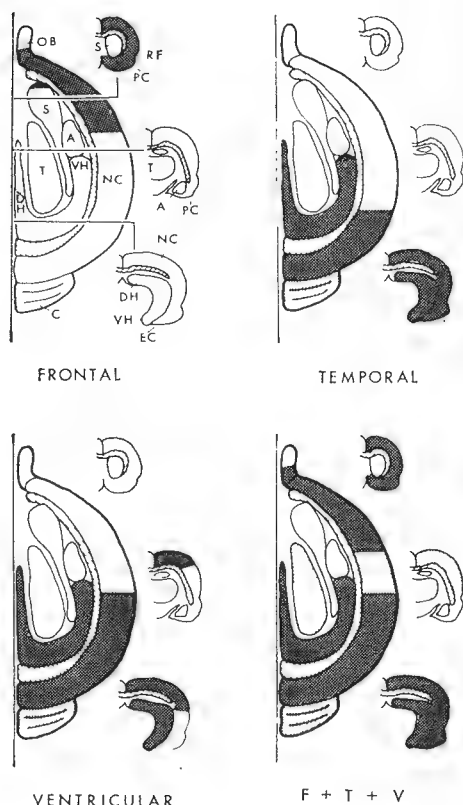


Fig. 1. Spread of fluorescein after intracerebral injection. The diagrams at the left indicate structures viewed from the top after removal of a horizontal section of the hemisphere; at the right, cross (frontal) sections of the hemispheres at the level indicated in the diagram for frontal injections. Relative intensity of staining is indicated by relative density of stippling. With all injections there was intense staining, not shown in the diagram, of the corpus callosum (cross hatched). Abbreviations: *A*, amygdaloid nucleus; *C*, cerebellum; *DH*, dorsal hippocampus; *EC*, entorhinal cortex; *NC*, neocortex; *OB*, olfactory bulb; *PC*, piriform cortex; *RF*, rhinal fissure; *S*, corpus striatum; *T*, thalamus; *VH*, ventral hippocampus; *F + T + V*, frontal + temporal + ventricular injections.

which shows the number of animals in which memory was lost, impaired, or retained after puromycin injection. In the legend of the table, the means and standard deviations of the percentage savings in retention are given for the three categories of memory. Percentage

Table 1. Effects of different sites of injection of puromycin on short- and longer-term memory. T, V, and F refer, respectively, to temporal, ventricular, and frontal injections, all given bilaterally.

Puromycin injections			No. of mice in which memory was:		
Site	Days after learning	Milligrams	Lost*	Impaired*	Retained*
<i>Short-term memory</i>					
T + V + F	1	0.03 to .06	7	0	0
T	1	.09	8	0	0
T	1	.06	14	3	1
V	1	.09	0	0	5
F	1	.09	0	0	5
V + F	1	.09	0	1	2
<i>Longer-term memory</i>					
T + V + F	18 to 43	0.03 to .06	7	0	0
T	11 to 35	.06 to .09	0	0	7
V	12 to 38	.06 to .09	0	0	3
F	16 to 27	.06 to .09	0	0	3
V + F	28	.06 to .09	0	2	2
V + T	28 to 43	.09	1	1	2
T + F	28	.09	0	0	3

* For the 37 mice with loss of memory, the means and standard deviations for percentages of savings of trials and of errors were respectively 1 ± 3 and 2 ± 6 ; for the seven mice with impaired memory, 26 ± 29 and 39 ± 12 ; and for the 33 mice with retention of memory, 90 ± 14 and 90 ± 9 . Negative savings in the group with lost memory have been designated zero so that the mean for this group is an overestimation of savings.

savings in retention tests were calculated for both trials and errors by subtracting the number to criterion in the retention test from the number to criterion in the learning experience, dividing by the number in the learning experience, and multiplying by 100. Retention tests were given usually 3 days after puromycin, to allow ample time for recovery of the animal. At the time of testing, any weight loss had commonly been regained and feeding, general locomotor activity, and reactions to the maze were normal.

Our first observations were made on mice trained to criterion on one arm of the maze and injected with puromycin 1 day later (Table 1, "Short-term memory"). After combined bilateral temporal, ventricular, and frontal injections, retention tests showed that memory of the training experience had been completely lost. An effort was then made to localize this effect. Memory was also completely lost with high consistency when puromycin was given in bilateral temporal injections. By contrast, bilateral frontal, ventricular, or combined frontal and ventricular injections were essentially without effect.

Table 2. Effect of bilateral temporal injections of puromycin on memory of increasing age. Each injection contained 0.09 mg of puromycin.

Injections: days after learning	No. of mice in which memory was:		
	Lost*	Impaired*	Retained*
2	3	0	0
3	4	0	1
4	0	1	1
5	0	1	2
6	0	0	3

* For the seven mice with loss of memory, the means and standard deviations for percentages of savings of trials and of errors were respectively 1 ± 4 and 0 ± 0 ; for the seven mice with retention of memory, 85 ± 19 and 93 ± 7 . In one mouse with impaired memory the percentages of savings for trials and errors were respectively 38 and 20; for the other, 39 and 55.

The next series of observations was made on mice trained to criterion and injected with puromycin 11 to 43 days later (Table 1, "Longer-term memory"). Only combined, bilateral temporal, ventricular plus frontal injections consistently destroyed memory in these animals. Bilateral temporal or frontal or ventricular injections were without effect. Three combinations of two injections (combined ventricular and temporal, or ventricular and frontal, or temporal and frontal) into each hemisphere were

Table 3. Differential effect of bilateral temporal injections of puromycin on recent and longer-term memory. Each injection had a volume of 0.012 ml and contained 0.06 or 0.09 (experiment 71) mg of puromycin. Choices of the arm of the Y-maze by an animal after injection were scored as "1" if consistent with initial learning, and as "2" if consistent with reversal learning. For various reasons trials were continued irregularly beyond the ten originally planned.

Expt. No.	Animal No.	Initial learning: trials to 9/10 criterion	Reversal learning 3 weeks later: trials to 9/10 criterion	Choice of arm of Y-maze*
<i>Experimental animals</i>				
86	26A	13	22	1,1,1,1,1,1,1,1,1,1,1,1,1,1,1
86	24A	7	10	1,1,2,1,1,1,1,2,1,1,1,1,1,1,1,2,1,1,1
86	25A	8	10	1,1,1,1,2,1,1,1,1,2,1,2,2,1,2
86	22A	9	8	1,2,2,1,1,2,1,2,1,2,1,1,1,1,1,1,1,1,1
86	23A	13	4	1,1,1,1,1,1,1,1,1,1,1,1,1
71	49	22	9	1,1,2,2,1,1,1,2,1,1,1,1,2,1,1,1,1,1,1,1
86	27A	12	5	1,1,1,1,1,1,1,1,1,1,2,1
<i>Control animals</i>				
86	58A	10	14	2,2,2,2,2,2,2,2,2,2,2,2,2,2,2
86	60A	10	12	2,2,2,2,2,2,2,2,2,2,2

* The experimental animals made their choices 3 days after temporal injections of puromycin, which were given 24 hours after reversal learning. The control animals made their choices 4 days after reversal learning, no puromycin being injected. Neither group received shock.

without effect in the majority of animals, even though the total amount of puromycin in all but two of eleven of these was at the maximum level tolerated and twice that amount injected in six of the seven experiments with combined temporal, ventricular, and frontal injections. There was consequently a clear distinction between recent and longer-term memory; recent memory was lost when puromycin was introduced through temporal injections into hippocampi and caudal cortices, including the entorhinal areas, while loss of longer-term memory required puromycin additionally in a substantially greater part of the cortex and possibly in the thalamus also.

How long does it require for this modification of the locus of the effective memory trace? As shown in Table 2, bilateral temporal injections consistently destroyed memory 2 days after training but were consistently without effect 6 days after training. Results were variable at 3, 4, and 5 days. It consequently appears that the enlarged locus of longer-term memory in the type of learning experience we have used with the mouse becomes completely effective

in from 3 to 6 days, depending upon the individual animal.

We proceeded from these observations to experiments in which the animal received reversal learning 3 weeks after its first training in the Y-maze, that is, the mouse was first trained, for example, to move from the stem of the Y into its left arm; then 3 weeks later was retrained to move from the stem of the Y into its right arm. Was it possible to destroy memory of reversal learning 24 hours after reversal training, spare the longer-term memory of the initial training experience given 3 weeks earlier, and in consequence have the mouse perform the task for which it was first trained?

To test this possibility bilateral temporal injections were made 24 hours after reversal learning and 3 weeks after initial learning in seven animals. Shock was omitted in the retention trials 3 days after puromycin injection, since there was, within the design of the test, no right or wrong choice. As shown in Table 3, on testing for memory the first choice of all animals was consistent with the first learning experience, as were the large majority of subsequent

choices. In view of consistent results with numerous untreated animals on various schedules of learning and reversal learning, only two control animals (Table 3) were used in this series. All these untreated animals, in sharp contrast to the experimental group, made choices consistent with their second, or reversal, learning. Because the experimental animals were able to perform the older position habit efficiently and consistently, this experiment offers strong evidence that the effect of puromycin in destroying a recent habit is not due to disorganization or incapacitation of the animal.

A beginning has been made in testing for the specificity and reversibility of the puromycin effect. Numerous control injections of saline, of subliminal concentrations of puromycin, and of puromycin hydrolyzed at the glycosidic bond were without effect on memory. Most animals treated with effective doses of puromycin were demonstrated to be capable of relearning after loss of memory, though the process of relearning, particularly with high doses of puromycin, often required considerably more trials than in the initial training experience. This aspect of the effects of puromycin will be reported more extensively at a later time.

Although the effective locus of short-term memory clearly appears different from that of longer-term memory we cannot now define the difference with precision. It does appear that the area around the caudal rhinal fissure, likely entorhinal cortex, carries the short-term memory trace, since short-term memory was retained with ventricular injections but lost with temporal injections. The part played by the hippocampus will not become evident until experiments are performed which provide for exposure of the entire temporal cortex to puromycin while the hippocampus is spared.

Comment. The experimental work for V.C.1-5 was done at the University of Pennsylvania by Drs. L. B. and J. B. Flexner. The consultations begun in 1938 still continue, however, and have been very illuminating in comparing synthetic processes in E. coli with those in the mouse. Richard B. Roberts.

Similarly, we cannot state whether the locus of longer-term memory is confined to the cortex or whether other parts of the brain, principally the hippocampus, are also involved. It can only be said that our observations are consistent with the evidence and conclusions of others (4), that the hippocampal zone is the site of recent memory and, that an extensive part of the neocortex is concerned with longer-term memory.

It must be emphasized that our results, although apparently clear-cut in important particulars, should be interpreted at this time with caution. We are in the process of obtaining more precise information, for example, on the localization of puromycin after intracerebral injection. Histological studies on the cells of the hippocampus and cortex must be completed. Determinations must be made of the degree of suppression of protein synthesis, and, particularly in view of the negative behavioral results with subcutaneous puromycin (3), the possibility must be kept in mind that loss of memory after intracerebral injection of puromycin may be owing to effects not related to changes in protein synthesis. Further, it remains to be shown that other learning situations, currently being investigated, and other animals are comparable to the mouse in the training experience we have used (5).

References and Notes

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VI. RIBOSOMES 1964

A. Introduction

Six years ago there were less than a dozen papers on bacterial ribosomes. Mammalian ribosomes had not been clearly distinguished from the matrix of other material in the microsomal pellets. In fact, the name ribosome had not even been invented. Microsomes appeared to provide the site of protein synthesis, and microsomal RNA was assumed to be the template. The concepts of messenger RNA and polysomes had not yet been developed.

The experimental work described in sections III and IV was started in 1957. These findings need some reinterpretation in terms of present ideas. Much of the work was concerned with the flow of material from the medium, through various precursor stages, to the end products of synthesis. Techniques were devised to fractionate the cells, and precursor-product relationships were established. The flows along various pathways can be calculated from the quantities of cellular constituents and the growth rate. Finally, when the sites of synthesis can be enumerated, the synthetic rate per site can be estimated. These kinetic considerations furnish some insight concerning the factors that control the rates and provide a more intimate view of the synthetic process.

The data available at present are sufficient to allow such calculations for the synthesis of DNA, D-RNA, R-RNA, S-RNA, and protein.* The results are reasonable and consistent with the concepts of "messenger RNA" and polysomes. The interpretation of the synthesis of ribosomal protein and ribosome formation in terms of polysomes is less satisfying, and a slightly different mechanism is suggested (VI.K). Other elements of the synthetic processes remain obscure. Nevertheless, they deserve equal attention because an examination of the inadequacies and discrepancies of present theories often leads to the formulation of new hypotheses and experiments. These unsolved problems are considered in section VI.L.

*Terminology. R-RNA refers to the class of RNA molecules having the same composition as the RNA extracted from ribosomes. D-RNA refers to RNA molecules having an average composition similar to that of the DNA of the cell. D-RNA is identical to "messenger RNA" as originally defined by Jacob and Monod.¹ We prefer the term D-RNA because the term "messenger RNA" has been so frequently applied to any newly formed RNA that it has lost its original definition. It has become equivalent to "nascent RNA," and ambiguous because nascent RNA includes a large component of newly formed R-RNA. The terms eosome and neosome (defined in III.B.2) remain useful in discussing ribosome synthesis. Neosome describes a ribosome having an incomplete complement of protein. The term eosome as originally defined included both D-RNA and R-RNA components and was equivalent to nascent RNA. Here its use will be restricted to nascent R-RNA, the first precursor of ribosomes.

B. Precursor-Product Relationships

Various experimental techniques have been utilized to separate classes of nucleic acids, nucleoproteins, and their precursors, and kinetic measurements have indicated their interrelationships. Several such studies are described in detail in sections II and III, and the results are combined to give the schematic flow diagram of Figure 1.

FLOW DIAGRAM-NUCLEIC ACID SYNTHESIS IN *E. COLI*

Areas proportional to quantities

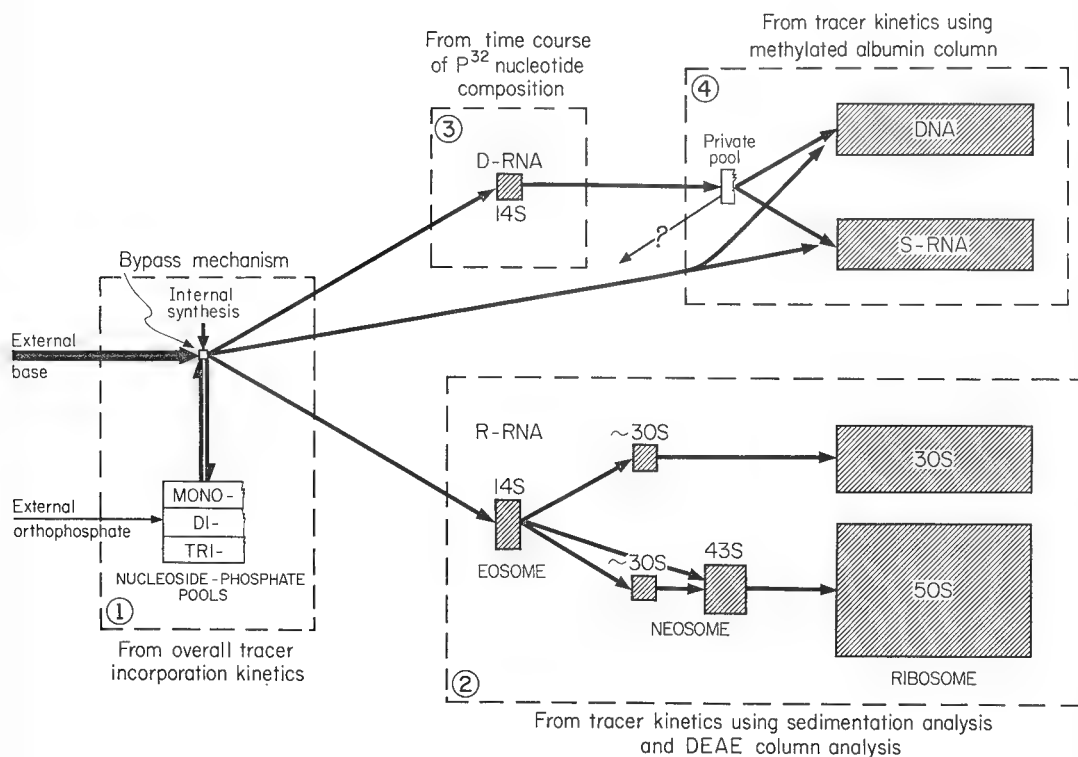


Fig. 1. Flow diagram: nucleic acid synthesis in *E. coli*.

The area indicated 1 shows the flow of materials through the pool of low-molecular-weight materials. The experiments and interpretations are described in detail in II.B. As was pointed out there, an understanding of the properties of the pool is essential in evaluating (or avoiding) the kinetic delays that may be introduced by the passage of external tracers through pools. Of particular practical importance is the pool bypass which allows immediate passage of exogenous bases into RNA and thereby permits accuracy in the kinetic analysis of subsequent steps.

Region 2 of the diagram was delineated from the kinetics of tracers passing through the fractions separable by sedimentation analysis and chromatography (III.B.2 and III.B.3). The eosomes and neosomes are clearly separable

precursors to the stable end products of synthesis, the ribosomes. The concurrent addition of protein is indicated in Figure 6 of III.B.3.

In the kinetic experiments described in III.B.2 the D-RNA component was not recognized as a separate entity. The component designated D-RNA (region 3) was inferred from measurements of the nucleotide composition of newly synthesized RNA (III.B.5). It was first isolated by selective removal from ribosomes (III.B.5) and later by means of the DNA-agar column (II.B.6). This component approximates closely the "messenger RNA" as defined by Jacob and Monod. It has a DNA-like composition, has a short lifetime in the cell, and very probably acts as the template for protein synthesis.

Region 4 of the diagram was explored by still other methods to distinguish S-RNA from other RNA and from DNA (III.B.5). Both S-RNA and DNA show kinetic delays which indicate that roughly one-half of their precursor material is derived from degraded D-RNA.

This flow, however, is not sufficient to account for the fate of all the D-RNA. Table 4 shows that 6.1×10^3 nucleotides per second are required for the synthesis of S-RNA and DNA. The flow of 5.0×10^3 nucleotides per second from D-RNA can supply one-half this material and leave 2.0×10^3 nucleotides per second to be used elsewhere. This flow is indicated by a question mark, as it is not directly observed but only inferred from the balance of flow rates. There seems little question that the material is reutilized for the synthesis of both D-RNA and R-RNA.

When cell extracts are fractionated by sedimentation, the apparent sedimentation coefficient of the newly formed RNA depends markedly on the magnesium concentration used (Figure 8 of III.B.2). Fractionations carried out at high concentration of magnesium (10^{-2} M) are difficult, if not impossible, to analyze, because the newly formed RNA is associated with larger particles and distributed throughout the profile. In contrast, analyses at low magnesium concentration show distinct, readily measurable peaks (Figure 8 of III.B.2). Corresponding to the 14S component observed in cell extracts is an 8S component of the RNA prepared by phenol (Figure 9 of III.B.2).

Other laboratories find newly synthesized RNA spread throughout a wide range of sedimentation constants.² At this time it seems likely that an artifact (degradation to 14S) made analysis easier in our experiments. Although 14S (and the corresponding 8S) objects were reproducible and well suited for kinetic analysis, no theoretical significance should be attributed to the size, because larger sizes are frequently observed.

The further events in the history of ribosomes beyond their initial synthesis as 30S and 50S particles are difficult to discern. Sedimentation analysis by itself shows little, since eosomes adhere to 70S particles (Figure 7 of III.B.2). Separation of large and small particles by sedimentation followed by frac-

tiation on DEAE to remove adherent nascent RNA indicates exchange between 30S and 50S ribosomes and larger particles.

The 100S ribosomes represent a simple dimer of the 70S particles, but they are seldom a prominent component in extracts of growing cells. The more frequently observed "85S" component is a much more complex phenomenon (III.D.1).

The proportion of 70, 85, and 100S groups that appear in the ultracentrifuge patterns depends on the metabolic state of the cell (III.A.1, III.C.5). Possibly the distribution is related to the state of organization of the ribosomes which existed in the cell at the time of breakage. Intact polysomes do not survive drastic methods of rupturing the cell wall, but some linkages might remain. Also, the proportion of nascent RNA and nascent protein might influence the formation of larger associations in vitro. A repetition of the earlier studies of ribosome patterns in different metabolic states, but using gentle methods of breaking to preserve polysomes, should be informative.

C. Quantities of Cellular Materials

Measurements of the quantities of cellular components are usually expressed in terms of milligrams per gram tissue. When the molecular weight is known, these numbers can be converted directly to micromoles per gram. For cellular components whose molecular weight is not known with any precision it is permissible to choose reasonable and convenient numbers. In Tables 1-9 the D-RNA templates are assumed to be the same size as the R-RNA of a 30S ribosome, i.e., 1700 nucleotides. The corresponding protein (assuming a coding ratio of 3) would contain 566 amino acids, which seems unreasonably large for monomer units. Accordingly it has been assumed that the average template of 1700 nucleotides forms 2 protein units of 283 amino acids (molecular weight about 31,000). The average structural gene is therefore taken to contain 850 nucleotide pairs.

The molecular weight of ribosomal proteins has been estimated to be about 25,000.³ Also the total protein content of a 30S ribosome is known. It is assumed that the 30S particle contains 12 units of protein each of molecular weight about 25,000. Ribosomal protein is therefore assumed to contain 239 amino acids, giving a molecular weight of 26,150.

Table 1 gives values for the nucleic acid and protein content of bacteria. The quantities listed in the first line are used as the basis for further calculations. For considerations of the detailed operations of the cell these values can be made more meaningful by expressing them in terms of molecules per cell. As a gram of dry cells contains roughly 4×10^{12} cells, multiplication by the conversion factor 1.5×10^5 is adequate for most purposes.

For calculating rates of synthesis per site it is more convenient to convert to molecules per bacterial chromosome (i.e., per ideal cell which contains a single complement of DNA). This conversion requires knowledge of the molecular weight of the bacterial chromosome.

Recent experiments⁴ have shown that the DNA of bacteria can be extracted in very long strands, and the length of the observed strands sets a lower limit to the size of the chromosome, which is 2.8×10^9 .

Cowie and McCarthy⁵ have measured the fraction of *E. coli* DNA that is homologous to a known length of λ phage DNA. This ratio indicates a molecular weight of 5.1×10^9 , or 8×10^6 nucleotide pairs, for the bacterial chromosome. Unfortunately, this method has an uncertainty that has not yet been resolved. A randomly phased population will contain cells in all stages of DNA replication (Figure 2). The average DNA content per nucleus will be $1\frac{1}{2}$ chromosomes if synthesis proceeds uniformly. If there is a definite point (P in Figure 2) on the chromosome at which synthesis is initiated, a gene located near the initiation point (A in Figure 2) will have 2 copies per nucleus. Another gene (C in Figure 2) located near the terminal end of the chromosome will show only 1 copy per nucleus. Accordingly the value of 5.1×10^9 will be correct if the stretch of DNA homologous to the λ phage is located centrally (B in Figure 2) or if there is no definite point of initiation. Depending on the location of the homologous region the molecular weight could vary from 3.7×10^9 to 7.4×10^9 , or from 6×10^6 to 12×10^6 nucleotide pairs.

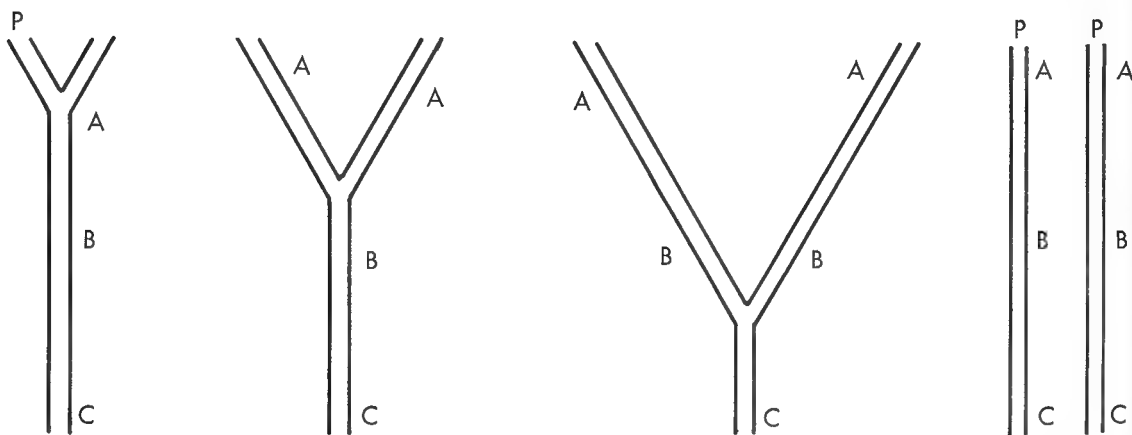


Fig. 2.

Kjeldgaard⁶ has observed 7 to 8×10^{-15} g of DNA per nucleus for *Salmonella typhimurium*. Correcting for the state of replication, this corresponds to 4.6 to 5.3×10^6 nucleotide pairs per genome. The value 6×10^6 pairs is used

TABLE 1. Nucleic Acid and Protein Content of Bacteria*

	RNA	DNA	Protein
Micromoles of nucleotides or amino acids per gram dry weight	650	120	5130
Average molecular weight of monomer [†]	325	309	109.5
Milligrams of nucleotides or amino acids per gram dry weight	211	37	560
Milligrams of nucleotides or amino acids per gram dry weight, data for <i>S. typhimurium</i> , generation time 50 minutes [‡]	220	35	740

*Data for *E. coli* from CIW Publication 607.

[†]Without water of hydrolysis.

[‡]From p. 63 of Kjeldgaard.⁶

TABLE 2. Nucleic Acid Content of Ideal Cell

Molecular weight <i>E. coli</i> DNA	3.7×10^9
1 molecule contains	1.2×10^7 nucleotides
1 gram <i>E. coli</i> contains (120 μ mole DNA nucleotides)	7.2×10^{19} nucleotides
Ideal cells (1 molecule DNA/cell)	6.0×10^{12} /g dry weight
RNA nucleotides/cell (650 μ mole RNA/g)	6.5×10^7

TABLE 3. RNA Components*

Component	Per Cent of RNA	Nucleotides/ Cell	MW	Nucleotides/ Molecule	Molecules/ Cell
Total RNA	100	65×10^6	---	---	---
S-RNA	20	13×10^6	2.8×10^4	86	1.5×10^5
D-RNA [†]	1.3	0.85×10^6	5.5×10^5	1700	5.0×10^2
Eosomes [†]	2.6	1.7×10^6	5.5×10^5	1700	1.0×10^3
Neosomes [‡]	7	4.5×10^6	1.65×10^6	5100	8.8×10^2
Ribosomes [‡]	69	45×10^6	1.65×10^6	5100	8.8×10^3

*For cells growing in glucose-salts medium; growth rate is 2 per cent per 100 seconds.

[†]RNA assumed to be same size as RNA of 30S ribosomes.

[‡]Ribosomes and neosomes calculated in terms of 70S units.

TABLE 4. Rates of Nucleic Acid Synthesis*

Component	Molecules/Cell	Molecules/Second	Nucleotides/Second
DNA	1	1/3465	3.5×10^3
S-RNA	1.5×10^5	30	2.6×10^3
D-RNA	5.0×10^2	3.0	5.0×10^3
Eosomes	1.0×10^3	6.0	1.0×10^4
Neosomes	8.8×10^2	2.0	9.9×10^3
Ribosomes	8.8×10^3	1.8	9.0×10^3

*Based on growth rate of 2 per cent per 100 seconds, or doubling time of 58 minutes, for RNA; linear rate for DNA.

TABLE 5. S-RNA Synthesis

	Nucleotides/ Sec/Site	Copies/ Generation
A. 40 independent sites	65	2700
B. 4 independent sites each producing 10 S-RNA molecules divided later	650	2700
C. 4 independent sites operating at appropriate rates to give observed abundances of S-RNA		
2 sites	950	3800
1 site	475	1900
1 site	240	950

TABLE 6. Ribosomal RNA Synthesis

No. of Sites	Size	Rate/Site, nucleotide/sec	Time Required, seconds
10*	70S	1000	5.1
10†	50S	666	5.1
10†	30S	333	5.1
12‡	50S	555	6.2
6‡	30S	555	3.1
8§	50S	833	4.1
12§	30S	277	6.1

*Unitary process.
†Equal numbers of sites for 30S and 50S.
‡Number of sites chosen to give equal rates of nucleotide addition.
§Suggested by data of Yankofsky and Spiegelman.¹¹

TABLE 7. RNA Products of DNA

Per Cent of DNA	No. of Sites	Copies/Site/ Generation	Product
0.04	40	2700	S-RNA
0.8	10	680	Ribosomes
1	70	30-200	Templates for abundant proteins such as fully induced en- zymes
30	2100	ca. 5	Templates for partially repressed enzymes
50	3500	1	Templates for fully repressed enzymes or control genes

TABLE 8. Synthetic Rates in Different Growth Conditions

Doubling time, minutes	180	60	20
Nucleotides per Site per Second			
DNA	1170	3500	10,500
R-RNA, 10 sites	110	1000	9,000
S-RNA, 4 sites	220	650	1,950
D-RNA*			
active sites	33	100	300
repressed sites	0.16	0.5	1.5
D-RNA†			
active sites	11	100	900
repressed sites	0.06	0.5	4.5

*D-RNA product assumed to be 1700 nucleotides in length; active sites, 200 copies per site per generation; repressed sites, 1 copy per site per generation independent of generation time.

†Activity of sites assumed to be proportional to growth rate.

TABLE 9

	Amino Acids per Cell*	Molecules per Cell†
<u>Quantities</u>		
Total protein‡	5.1×10^8	1.8×10^6
Ribosomal protein†	7.8×10^7	3.3×10^5
Nonribosomal protein§	4.3×10^8	1.5×10^6
<u>Synthetic rates (per second)</u>		
Total protein	1.0×10^5	3.6×10^2
Ribosomal protein	1.5×10^4	6.6×10^1
Nonribosomal protein	8.6×10^4	3.0×10^2

*Average molecular weight 127.5 or 109.5 in protein.

†Ribosomal protein 239 amino acids, molecular weight 26,150.

‡Total protein: 59% cellular carbon; 5130 μ mole amino acid per gram.

§Average nonribosomal protein calculated for 283 amino acids, molecular weight 31,000.

as the basis for calculating Table 2. It is particularly convenient because the factor converting micromoles per gram to molecules per cell becomes 1×10^5 .

Table 3 shows the quantities of the various RNA components. The procedures for separating them and measuring their relative quantities are described in section III. The proportion of ribosomes depends on the growth rate, and so these values do not apply to cells grown at different rates in other media.

D. Rates of Nucleic Acid Synthesis

The rates of synthesis calculated in Table 4 are based on a growth rate of 2 per cent per 100 seconds, which corresponds to a doubling time of 3465 seconds. The DNA rate is based on the assumption that nucleotide addition proceeds at a uniform rate throughout the entire period. The rates of the stable RNA components (S-RNA and ribosomes) are calculated to give a 2 per cent increase per 100 seconds. The intermediates (eosomes and neosomes) themselves must increase at 2 per cent per 100 seconds and must also supply the material needed for their products. Finally, the D-RNA rate is taken to be one-half the rate of eosome synthesis.

E. Sites of Nucleic Acid Synthesis

DNA. The elegant radioautographs of Cairns show only one point of synthesis along the DNA molecule.⁴ Hence the individual strands must accumulate nucleotides at a single point at one-half the total rate of 3500 per second. Bacteria growing in broth with a 20-minute generation time must have a rate three times higher. These experiments, which exclude multiple sites of synthesis along the DNA molecule, demonstrate that rates of 5000 nucleotides per second at a single site are possible.

S-RNA. The portion of the DNA that can hybridize with S-RNA has been measured.^{7, 8} These experiments show that 0.02 per cent of the DNA, or 2.4×10^3 nucleotides, are in sequences complementary to S-RNA. Such a short stretch of DNA would be able to act as template for only 28 different types of S-RNA molecules each containing 86 nucleotides. Sueoka⁹ has observed 30 different fractions in chromatographic separations. Perhaps the S-RNA genes are located near the terminal end of the chromosome (see Figure 2) and are represented less frequently than the average in the DNA of a randomly phased culture. In this event the actual quantity of DNA would be 1.5 times greater, sufficient to act as template for 42 types of molecules.

Table 5 lists the rates of S-RNA synthesis as calculated according to various models of the mechanism. In line A it is assumed that equal quantities of the forty types of S-RNA are made at forty independent regions of the DNA. In line B it is assumed that four independent regions (of equal activity) provide

four sites of synthesis and synthesize long RNA molecules which are subsequently chopped into ten pieces, of S-RNA size.

Countercurrent separation of S-RNA indicates that the different fractions differ in abundance.¹⁰ In C, the four regions are assumed to have different activities to account for the differences in abundance found by fractionation.

R-RNA. Measurements have been made of the fraction of DNA complementary to ribosomal RNA. Yankofsky and Spiegelman have found (in Bacterium megaterium) 0.18 per cent complementary to the RNA of 50S ribosomes and 0.14 per cent complementary to the RNA of 30S ribosomes.¹¹ McCarthy and Bolton have found 0.4 per cent of E. coli DNA complementary to ribosomal RNA.¹² Such a quantity would provide sites for the RNA of 9 to 10 70S ribosomes, or (if divided in the 18/14 ratio observed by Yankofsky and Spiegelman) for 12 sites for the RNA of the 30S ribosomes and 8 sites corresponding to 50S ribosomes.

Since the 30S and 50S particles are made in equal numbers it is far more attractive to believe that they are made in a unitary process. In mammalian cells, nascent R-RNA first appears in large molecules which subsequently separate into the 16S and 28S molecules found in the ribosomes.^{13, 10} Taking this view, the rate is about 0.2 molecule per site per second (i.e., 5.1 seconds per molecule) and the rate of nucleotide addition is 1000 per second, roughly one-half the rate required for DNA synthesis (Table 6). There appears to be no need to assume any self-replicating mechanism for the synthesis of R-RNA.

Once again, the number of sites is uncertain because genes specifying R-RNA might be represented more or less frequently than the average in the DNA of a randomly phased culture. Accordingly the number of sites calculated here may be too high or too low by as much as a factor of 1.5.

D-RNA. The synthesis of ribosomal RNA and S-RNA requires less than 1 per cent of the DNA, leaving the rest available to act as template for D-RNA. The rate of D-RNA synthesis can be determined from its equilibrium level and time constant, or from the observation that one-third of the newly synthesized RNA is D-RNA and two-thirds ribosomal RNA. This rate of 5.0×10^3 nucleotides per second is approximately $1\frac{1}{2}$ times the rate of DNA synthesis. In DNA synthesis each nucleotide is copied once per generation, and so the average rate of making D-RNA copies is $1\frac{1}{2}$ per generation.

A number of lines of evidence show that only one strand of the DNA is complementary to D-RNA. Melting curves of D-RNA indicate no sign of double-stranded molecules.^{12, 14} A DNA-agar column becomes saturated with D-RNA when one-half its weight of D-RNA has formed complementary strands. The remaining half, however, is still available to form duplexes with additional DNA.¹²

As double-stranded DNA provides only one template for D-RNA, the average number of copies produced per template is 3 per generation. Furthermore, there is a wide distribution in the number of D-RNA molecules of different types. When D-RNA is incubated with DNA-agar, only a small quantity is needed to saturate some of the DNA sites but very high concentrations are needed to achieve the full saturation of 50 per cent. From these data the relative activities of different parts of the DNA template can be calculated.¹² Figure 3 shows the distribution of frequencies of RNA molecules if the genes are ordered according to their activity. Although no great accuracy is possible in this type of analysis (since it depends on the slope of the saturation curve) it is clear that a small fraction of the templates produce most of the D-RNA, and a large majority of the templates have a minimal activity.

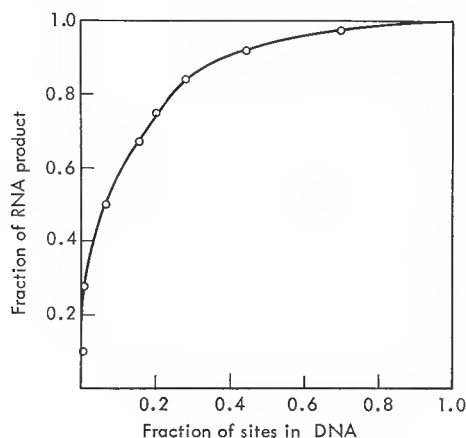


Fig. 3.

As the average is 3 copies, 50 per cent making only 1 copy would contribute 17 per cent of the product. Figure 3 shows roughly this value. The number 1 has a special significance; a possible association with DNA replication which must occur once per generation is difficult to ignore. At the other extreme less than 1 per cent of the DNA contributes 10 per cent of the D-RNA, giving an average rate greater than 30 copies per generation. In fact, the curve indicates that the maximum rate may be as high as 200. In summary, taking 850 nucleotide pairs as a reasonable size for an average gene, *E. coli* contains sufficient DNA for 7000 genes. A few of these appear to produce 200 D-RNA copies per generation. Roughly 70 have an average rate of 30 per generation, but the majority produce only 1 copy per generation.

This range of activities corresponds to the known range of expression of induced enzymes. The minimum value of 1 copy per generation might be attributed to the enzyme-forming systems that are not induced or are fully repressed. Intermediate values correspond to partially repressed systems, and the maximum values correspond to fully induced enzyme-forming systems. Table 7 summarizes the production rates that can be attributed to various portions of the DNA.

It must be emphasized that these calculations give average production rates; no direct information is available to indicate the actual time required for the synthesis of any one molecule. The rate of 1 copy per generation might mean that the molecule was slowly assembled during the whole generation. It is more likely, however, that the molecule is synthesized in a single rapid event that occurs only once per generation. Also, when multiple copies are produced, the synthetic events might be spaced throughout the generation or they might occur in a burst of activity at one particular phase of the DNA replication.

F. Limitations on Synthetic Rates

The mechanisms controlling nucleic acid synthesis remain obscure. The most likely factors to limit the rates are the concentrations of substrates, polymerizing enzymes, or templates. Furthermore, all three of the reactants may have active and inactive forms. Substrate molecules may require attachment to hypothetical carriers; enzymes may require cofactors or be inhibited by combination with other cellular components; and the templates may be present but blocked by other molecules or not in the required configuration. The rate calculations of the preceding sections provide some new information that is useful as an indication which of these factors may be predominant.

Table 8 summarizes the rates calculated above and indicates how these rates depend on the growth rate. Kjeldgaard⁶ has shown that the proportion of R-RNA increases with the growth rate but S-RNA maintains the same ratio to DNA. In other words, the genes specifying the R-RNA produce more copies per generation at the higher growth rates, but the genes specifying S-RNA produce a constant number of copies per generation.

The variation of D-RNA production with growth rate has not been measured directly, and the rates have been calculated for two alternatives. It seems likely, however, that the D-RNA must remain proportional to R-RNA. At a generation time of 60 minutes the proportion of D-RNA has been measured and found to be just sufficient to utilize all the ribosomes for polysome formation (section VI.I). When the generation time decreases to 20 minutes, the ribosome content per cell increases by a factor of 3 and the rate of protein synthesis also increases by a factor of 3. Presumably the polysome content also increases by a factor of 3, requiring an equal increase in the production of D-RNA.

The increase could be achieved by an increase in the proportion of active genes, without any increase in the number of copies per generation produced by genes already active. This alternative seems unlikely, because the rapid growth is produced by a richer medium, which tends to repress, not induce. Furthermore, if the fully induced genes do not vary the number of copies per generation, the proportion of their protein product should decrease with growth rate. The most likely situation is that D-RNA production is as responsive as R-RNA to changes in the growth rate (Table 8).

The rate of R-RNA synthesis may be limited by the supply of nucleotides. The prompt incorporation of exogenous bases shows that the cell contains less than 5 seconds' supply of nucleotides in a form ready for incorporation into RNA (II.C.2, II.C.3). The corresponding intracellular concentration is 5×10^{15} molecules per milliliter for any one base. The rate at which molecules can diffuse to a synthetic site has been calculated by Pollard.¹⁵ Fifteen hundred molecules per second could diffuse to a hemisphere of 5 Å radius if the concentration is 5×10^{15} per ml. Nucleotides of any one species should diffuse to the sites of R-RNA synthesis at a rate of 1000 per second to be available when needed even though the rate of incorporation is fourfold less. Considering the uncertainties in the estimation of the concentration and the radius of the synthetic sites, these calculations only show that the synthetic rates lie in the range where diffusion limitations might be expected.

The rate of R-RNA synthesis increases abruptly* when cells are shifted to a richer medium.⁶ The time of the transition is too short for any major change in the level of enzymes indicating that the cells already contained sufficient polymerase to allow a higher rate of synthesis. An increase in the supply of nucleotides is the apparent reason for the increased rate of synthesis, but activation of existing enzyme molecules cannot be ruled out as an alternative interpretation.

The rate of DNA synthesis is even higher and it also lies in the range where the supply of substrate might be limiting. A shift to a richer medium does not cause an immediate increase in the DNA rate, however; synthesis of new enzymes seems to be required.

The limitations on the rate of S-RNA synthesis seem to be quite different. In particular, the ratio of S-RNA to R-RNA depends on the growth rate indicating that the same general controls such as the supply of nucleotides and polymerase cannot govern both. Also, the same number of S-RNA copies (per generation) are formed under widely different metabolic conditions. This suggests that the sites for S-RNA synthesis operate a fixed number of times per generation and may be coupled in some unknown way to the state of DNA replication.

*A decrease in the rate of D-RNA synthesis might be expected on theoretical grounds when cells are shifted to a richer medium. A number of enzymes are repressed, and the synthesis of their templates should decrease. Such an inhibition has been reported by Gros et al.¹⁶ Kjeldgaard did not observe this effect in measurements of the total rate of RNA synthesis.⁶ Neither have we been able to repeat these results. If the repression does occur it seems to be overcompensated by the increased synthesis of ribosomal RNA and not observable without separation of the two components. A possible explanation of this discrepancy in experimental results is that the casein hydrolysate used by Gros et al. contained adenine, which diluted the C^{14} adenine used as tracer.

The synthesis of D-RNA seems to be limited by the state of the template. The low rate of 1 copy per generation can hardly be attributed to substrate or enzyme limitations when the same cell can synthesize R-RNA at 1000 times these rates. Finally, the 200-fold changes in rate that occur when inducers are added can only be attributed to limitations at the template.

When cells are transferred from a rich to a poor medium the rate of RNA synthesis is extremely low.⁶ During this period what little RNA is made has a DNA-like composition.¹⁷ This phenomenon might be attributed to a block in the synthesis of R-RNA. If, however, the nucleotide supply is so low that the availability of the DNA to act as template is no longer rate-limiting, the product would be DNA-like simply because of the preponderance of sites that specify D-RNA. Thus there is no need to look for a mechanism that prevents R-RNA synthesis. Its production will be negligible if all sites compete equally for a small supply of nucleotides.

In summary, a consideration of the synthetic rates shows that the different kinds of nucleic acids are formed at widely different rates and that these rates respond differently to changes in the metabolic conditions of the cell. Some of the rates may be limited by the supply of nucleotides or polymerase, but others must be limited by the state of the template.

G. The Role of Ribosomes in Protein Synthesis

The preceding sections have dealt with ribosomes, ribosome synthesis, and RNA synthesis. The facts discussed there provide no direct indications of the part ribosomes may play in directing the formation of protein in accord with the information carried by DNA. However, they provide necessary conditions that must be met by any theory of information transfer.

The correlation of facts relating to the flow of material with another set of facts relating to the flow of information is extremely difficult. In most kinetic experiments only the average flow of material into all proteins or all ribosomes can be measured. Furthermore, there may be no immediate alteration in the flow if some of the products are faulty and nonfunctional. In contrast, the experiments dealing with information usually measure one particular function such as enzymic activity, and the rate of synthesis of the particular functional enzyme may differ markedly from the average rate of protein synthesis.

At present the most precisely formulated theory of the role of ribosomes in information transfer is the messenger hypothesis of Jacob and Monod.¹ The kinetics of enzyme induction in bacteria indicate that enzyme-forming units are unstable and that template material must be continuously supplied by the gene. However, the ribosomes which appear to be the sites of protein synthesis are stable. Accordingly, Jacob and Monod postulated that an unstable messenger RNA is produced by the gene and associates temporarily with ribosomes to act as template for protein synthesis.

They predicted that cells should therefore contain an RNA fraction having the following properties: (a) an average molecular weight of 5×10^5 or more, (b) a base composition reflecting the base composition of DNA, (c) a capacity to associate with ribosomes, and (d) a high rate of turnover.

Template material, however, is difficult to identify. At best, kinetic and compositional studies identify material having the properties postulated for messenger. Hybrid formation is not an adequate criterion, as it indicates only a specific relationship to DNA which has been found in all classes of RNA. Template material might be identified by its ability to direct the synthesis of protein in cell-free systems if the properties of the cell-free system were known to be the same as those of the intact cell. Such an assay would be subject to many uncertainties, as slight degradation or secondary structure might obscure the properties of true template material.

Of the various classes of RNA only D-RNA can provide the templates for the bulk of the cellular protein. S-RNA is far too small to act as template. R-RNA is complementary to only 0.4 per cent of the DNA and thus can contain only a sequence of 24,000 nucleotides. With a coding ratio of 3, sequences of 8000 amino acids could be specified. This is more than adequate to act as template material for the 20 proteins of some 250 amino acids isolated from ribosomes by Waller and Harris,³ but it is much too low to specify the variety of enzymes found in bacteria. Thus the identification of the template material seems to be established by elimination. Moreover, the special templates that have been investigated, such as templates for virus protein and for β -galactosidase, have turned out to be D-RNA.^{18, 19}

H. Rates of Protein Synthesis

As is indicated above, the evidence is strong that D-RNA provides the templates for protein other than ribosomal protein. If, in fact, D-RNA also acts as template for ribosomal protein the rates of protein synthesis calculated below will be 15 per cent low because ribosomal protein (15 per cent of the total) is not included.

The nonribosomal protein contains roughly 4.3×10^8 amino acids, and to maintain a growth rate of 2 per cent per 100 seconds amino acids must be polymerized at a rate of 8.6×10^4 per second. The quantity of D-RNA available as template material contains 8.5×10^5 nucleotides, enough for 1.0×10^3 templates of 850 nucleotides each.

Assuming that all the D-RNA is active in protein synthesis, the rate of amino acid incorporation is then 86 amino acids per second per template, and 3.3 seconds would be required to complete each peptide strand of 283 amino acids. If the templates are in fact larger, the average rate of amino acid incorporation per template must be higher, as they are fewer, but the average time

to complete the polypeptide strand remains the same. As the D-RNA is degraded with a time constant of 150 seconds, the average number of peptide strands produced during the lifetime of the template is 45.

I. Polysomes

The foregoing estimates give the rate of synthesis per template molecule. If each template molecule provides only a single synthetic site, the rate per site is the same as the rate per template. Recently the theory has been introduced that the strands of D-RNA may attach to several ribosomes, forming what is designated as polyribosome or polysome,^{20, 21} the synthetic sites being located at the points of attachment.

This theory is based on sedimentation analyses showing newly formed protein associated with relatively heavy particles and on electron microscope pictures showing groups of ribosomes. It is also attractive on other grounds. In fact, several clues pointed to the existence of polysomes before they were observed (see section VII).

The apparent excess of ribosomes is accounted for by the polysome theory. Goodman and Rich report the association of five ribosomes with the template for the 150 amino acid polypeptide chain of hemoglobin. Using this figure of 90 nucleotides per ribosome the D-RNA is sufficient to occupy 9.4×10^3 of the 8.8×10^3 ribosomes (Table 3). The precision of this agreement is probably fortuitous, but Schaechter²¹ finds one-half the total ribosome material of B. megaterium still associated as polysomes after the cells are broken. The fraction might well be higher in the intact cell.

According to the polysome theory, free ribosomes attach to the template RNA and pass along its length as the synthetic process occurs. The polypeptide product accumulates on the ribosome. On reaching the end of the template the ribosome is released and subsequently the polypeptide is detached from the ribosome. The total number of ribosomes, R , will then be the sum of three classes: the free ribosomes, R_F ; the ribosomes engaged in synthesis, R_S ; and the ribosomes free of the template but still entangled with the completed polypeptide, R_P . Also the average time per synthetic event, T , may be divided into corresponding periods: T_F , T_S , and T_P .

If the average length of the product polypeptides, L , is known, T is given by the equation $\phi T = RL$. ϕ , the flux of amino acids, is 8.6×10^4 amino acids per second (Table 9). If we assume that $L = 283$ (template of 850 nucleotides, coding ratio of 3), and take $R = 8.8 \times 10^3$ (Table 3), then $T = 29$ seconds.

Nascent protein, i.e., newly formed protein still associated with ribosomes, might occur in two forms: completed strands still attached to free ribosomes, and unfinished strands still in the synthetic process. The quantities of these

two components would be R_pL and $\frac{1}{2}R_S L$ if the synthetic process continues at a constant rate along the template. Schaechter's data²¹ show only a very small fraction of the nascent protein associated with 70S particles. Although conditions might be different in the intact cell it seems reasonable to assume R_p is very small. Hence T_p is very short, since

$$\frac{T}{R} = \frac{T_F}{R_F} = \frac{T_S}{R_S} = \frac{T_P}{R_P} = \frac{L}{\phi}$$

Values for the quantity of nascent protein are 3 to $5 \times \phi$ (IV.A.1) and 10ϕ (III.B.3). Taking the higher value, since loss of nascent protein is a likely cause of the discrepancy, and assuming that $T_p = R_p = 0$:

$$\frac{1}{2}R_S L = 10\phi$$

Since $\phi T = RL$,

$$R_S/R = 20/T = 70 \text{ per cent}$$

and

$$T_S = 20 \text{ seconds}$$

This number is consistent with Schaechter's observation that one-half of the ribosomal material is associated with polysomes.

No great significance should be placed upon this agreement, however, because it depends on the estimate $L = 283$. This number was chosen because the template is of reasonable size and the product protein lies in a common size range (molecular weight 30,000). If the product protein differs,

$$T \sim L^{+1}; \quad T_S \sim L^0 R_S \sim L^{-1}$$

The observed quantities of nascent protein are consistent, however, with polysome theory. In contrast, if nascent protein corresponded to half-filled sites in the nucleic acid template, we would expect only $1/2 \times 1/3 \times 8.5 \times 10^5 = 1.2 \times 10^5$ amino acids (Table 3), or 1.4ϕ .

For the assumed length of 283 amino acids to be completed in 20 seconds the rate of addition must be 14 per second. If the same template provided only a single site of synthesis the rate would be 86 per second. Thus the polysome theory allows considerably more time for the difficult process of selecting the proper amino acids for insertion into the growing chain.

J. The Synthesis of β -Galactosidase

The calculations of the preceding sections pertain to the synthesis of average templates and average proteins. They can be tested in more detail by applying them to the synthesis of one particular protein. β -Galactosidase is one of the most suitable examples for comparison with theoretical estimates because of the mass of data available concerning it and the kinetics of its synthesis. The cells produce a small quantity of enzyme without inducer. Upon induction, the rate rises rapidly, approaching a 200-400 times greater rate with a time constant of 150 seconds. In the induced cells the level of enzyme commonly reaches 2 per cent of the total protein.

A quantitative description of this process follows as a consequence of the following assumptions: (1) In the uninduced state the gene determining the structure of the enzyme belongs to the class of quiescent genes that produce only 1 RNA copy per generation. (2) Upon induction, the gene joins the class having the highest activity and produces 400 RNA copies per generation. (3) The lifetime of this template is the same as that of other D-RNA molecules. (4) This particular template polymerizes amino acids at the average rate.

The monomer unit of β -galactosidase has a molecular weight of 40,000 and thus contains 365 amino acids.²² Its template would therefore contain 1085 nucleotides. At the maximum rate of RNA transcription (1000 nucleotides per second) the first template could appear within a few seconds after adding the inducer even if several genes were transcribed in a unitary process. Thereafter, additional templates should be produced at the average rate of 400 per generation, or 1 every 9 seconds. The time constant for the degradation of D-RNA is 150 seconds (III.B.5; III.B.6). Therefore the quantity of template material per cell should increase as

$$N = N_0 (1 - e^{-t/150})$$

where N_0 is 17, the number of templates per cell in the induced state. This time course corresponds to the time course of the rate of enzyme synthesis reported by Pardee and Prestidge²³ and by Boezi and Cowie (IV.A.4).

The quantity of enzyme synthesized during the lifetime of the template can be calculated from the flow of 14 amino acids per second per site, estimated in section VI.I. Kiho and Rich have shown that this enzyme is made by a polysome complex containing about 12 ribosomes per template.²⁴ The total flux should then be 12×14 amino acids per second. If the template is fully active during its lifetime of 150 seconds, 25,200 amino acids could be polymerized into 70 monomer units. This should be the level of enzyme found in uninduced cells. After induction the quantity should be 400 times as high, or 1.1×10^7 amino acids, which is 2 per cent of the total.

The quantity of nascent enzyme, still attached to the ribosomes, can also be calculated. Each of the 17 templates present in induced cells should carry 12 ribosomes with enzyme strands in various stages of completion. If the average is $\frac{1}{2}$ of a complete strand per ribosome, then 3.7×10^4 amino acids should be bound in enzyme that is still under construction. This corresponds to the quantity of enzyme completed in 13 seconds of synthesis. Kiho and Rich observe enzyme activity corresponding to 0.8 second of synthesis in the polysome fraction.

Partially complete strands which have not finished the synthesis of the active site should have no enzyme activity whatsoever. Furthermore, the activity of ribosomal bound enzyme can be increased as much as sixfold by treatment with antibody (IV.A.3).²⁵ Thus the factor of 16 between the predicted quantity of partially formed enzyme and the observed activity of the nascent enzyme lies in the expected range.

In summary, the observed quantities of enzyme are in accord with the assumption that the cell produced 1 template per generation in the uninduced state. In view of the difficulties in estimating the number of enzyme molecules present from the observed activity of incomplete units, this assumption may provide the best available estimate of the number of enzyme-forming units.

K. The Synthesis of Ribosomal Protein

The theory that messenger RNA associates with a number of ribosomes to form polysomes provides a very satisfactory interpretation of many features of protein synthesis. Calculations based on this theory lead to reasonable results in accord with experimental observations (VI.I, VI.J). It is quite possible that ribosomal protein is formed in the same way and finds its way to associate with newly formed R-RNA after leaving the polysome complex. No experimental evidence exists to disprove this hypothesis. For a number of reasons, however, it is unsatisfying.

Ribosomal protein comprises about 15 per cent of the total; hence its synthesis might be expected to occupy 15 per cent of the ribosomes and 15 per cent of the D-RNA. Ribosomal protein has been resolved into roughly 20 components of molecular weight 25,000.³ Thus, a stretch of DNA containing 14,000 nucleotide pairs should be sufficient to specify the ribosomal protein. This quantity of DNA, about 0.2 per cent of the bacterial chromosome, would be required to produce 15 per cent of the D-RNA. Genes having an activity as high as this have been observed (VI.C), and it is possible that they are, in fact, the ones that specify ribosomal protein. If, however, they are allocated to ribosomal protein there will be few remaining genes of high activity to allocate for the production of other abundant proteins such as fully induced enzymes. It is, therefore, attractive to speculate that R-RNA might provide the templates for ribosomal proteins.

The fraction of the DNA complementary to R-RNA has been found to be 0.4 per cent (i.e., 0.8 per cent of the nucleotide pairs); thus, R-RNA has ample length to act as the template for ribosomal protein. But if nascent R-RNA competed with D-RNA in the formation of polysomes, roughly two-thirds of the ribosomes would be associated with R-RNA and not available for the synthesis of nonribosomal protein. Also, it has been shown that R-RNA is more tightly bound to particles than D-RNA is, indicating that the two types of complex are different (III.B.5). Finally, the R-RNA strand of a 70S ribosome is long enough to engage 57 ribosomes and might be expected to act as template 57 times or more as the ribosomes proceeded along its length. As a result, far too much ribosomal protein would be produced. This mechanism provides no natural way for the ribosomal protein to become associated with the nascent R-RNA to form neosomes and ribosomes.

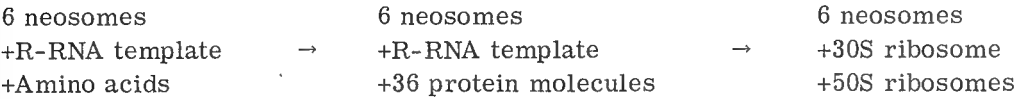
Many of these difficulties disappear if we postulate that neosomes (III.B.2) form a complex with nascent R-RNA and that this complex acts analogously to the polysome to synthesize ribosomal protein. Furthermore, we assume that R-RNA sufficient for a 70S ribosome is initially made as one piece, as this mechanism automatically ensures the production of equal numbers of 30S and 50S ribosomes. The split into the 16S and 23S portions found in ribosomes is assumed to take place after the RNA has served as template for ribosomal protein.

The 70S ribosome contains 5100 nucleotides and 8500 amino acids, one-third of each being in the 30S component and two-thirds in the 50S. Also, the protein is accreted in distinct stages (III.B.3). The 30S ribosome appears to be formed by the addition of two portions of 1433 amino acids, each being sufficient for 6 protein molecules of 239 amino acids. The molecular weight of these molecules would be 26,150, corresponding closely to the units estimated by Waller and Harris³ to have a molecular weight of 25,000. To indicate the compositions of the particles symbolically, the 30S neosome and the 30S ribosome are designated NP₆ and NP₁₂, respectively. The 43S neosome and the 50S ribosomes, which contain twice as much nucleic acid and protein, are designated N₂P₁₂ and N₂P₂₄.

The RNA of a 70S particle could serve as template for a polypeptide of 1700 amino acids. Thus one reading of the RNA would suffice to produce 6 molecules of ribosomal protein, the complement needed to make a 30S neosome or to convert a 30S neosome to a 30S ribosome.* If then the RNA of a 70S ribosome passed over six points of synthesis the appropriate quantity of protein would be formed to convert the naked RNA into a complete 70S ribosome.

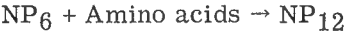
*The correspondence is not exact. An excess of 267 amino acids (16 per cent) is left over. Perhaps this apparent excess is due to an error in the observed protein/RNA ratio of ribosomes. If the protein were in fact 16 per cent greater, the ratio would be 40/60 instead of 37/63. Alternatively, the 16 per cent could be lost from the ribosomes, or not made.

Assuming that neosomes do provide the points of synthesis, the over-all reactions can be written:

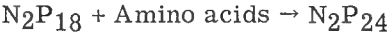


The reactions of the individual particles can be visualized in greater detail as follows:

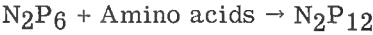
1. A 30S neosome (NP₆) passes along the R-RNA template and accretes 6 molecules of protein, sufficient to convert it to a 30S ribosome (NP₁₂).



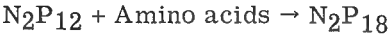
2. A 43S neosome lacking one complement of protein (N₂P₁₈) passes along the template and accretes the 6 molecules of protein needed to convert it to a 50S ribosome (N₂P₂₄).



3. The 43S neosome is produced by two similar reactions:



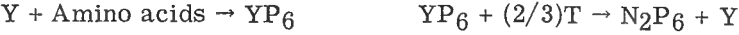
4. and



5. In the final reactions one particle (X) passes along the template and synthesizes the usual six molecules of protein. The protein products, however, remain entangled with one-third of the template (T) to produce a 30S neosome and X emerges unchanged.



6. Another particle (Y) passing through similar reactions remains entangled with two-thirds of the template to produce the N₂P₆ particle.



As X and Y are unchanged in the reactions they could be either 30S or 43S neosomes.

The exact details of the reactions probably have no significance, as the events are occurring in a complex in which identities may be lost. They do,

however, show that the postulated reactions are consistent with the known facts of ribosome synthesis (III.B.2 and 3), provided that the 43S neosomes include two classes, N_2P_{12} and N_2P_{18} , which are not resolved by sedimentation, and that the particle N_2P_6 is unstable when the cells are broken and disintegrates to $NP_6 + \text{RNA}$.

Assuming that the particles designated X and Y are 30S neosomes, to give equal numbers of N and N_2 units, the complex would contain 9 units of R-RNA in the form of neosomes compared with 3 units in the form of nascent R-RNA. The ratio observed in the cell is 7/2.6 (Table 3).

The quantity of nascent R-RNA is sufficient to make 333 of these aggregates per cell. The rate of formation of 70S ribosomes is 1.8 per cell per second when the generation time is 60 minutes. Thus the average time for the synthetic events is 185 seconds, which corresponds closely to the time constant of the nascent RNA.

The rate of synthesis of ribosomal protein is 1.5×10^4 amino acids per second. The 333 aggregates would provide 2000 sites of synthesis, and the rate per site would be 7.5 per second. The corresponding rate of nonribosomal protein synthesis (calculated on the basis of polysomes) is 14 amino acids per second per site.

This hypothesis makes very definite predictions by which it can be tested. The postulated aggregates contain RNA equal to that of four 70S ribosomes. Thus they can be expected to sediment at approximately 140S. They should contain all the nascent R-RNA and all the neosomes, 2.6 per cent and 7.0 per cent of the cellular RNA, respectively. A small peak does appear in this region of the sedimentation diagrams published by Schaecter²¹ and by Kiho and Rich.²⁴

The postulated peak should be made more apparent by appropriate use of tracers. At early times after the addition of labeled uracil to a growing culture, two-thirds of the nascent RNA should appear in this R-RNA peak compared to one-third distributed as D-RNA among the polysomes. At slightly later times the proportion should be even higher, as this peak should contain also the second ribosomal precursors, the neosomes. Being composed of R-RNA the peak could be distinguished from D-RNA by its base composition or by hybridization to DNA.

With labeled amino acids as tracers, the peak should not be prominent at very early times. Only 15 per cent of the label should be present, the other 85 per cent being distributed among the nascent proteins of the polysomes. The time constant of the nascent protein associated with the polysomes is short, and this component should saturate in 10-20 seconds. Radioactive proteins should continue to accumulate in the neosomes as their time constant is 450 seconds. The sedimentation diagrams of Kiho and Rich²⁴ show a peak of newly formed

protein in the appropriate region, but the time of exposure was too short (30 seconds) to maximize the relative specific radioactivity.

In short, if Nature is kind, one region of the sedimentation diagram should have very distinctive tracer kinetics. These can be predicted from the known flows of tracers into ribosome precursors (III.B.2 and 3). It is more likely, however, that the complex will be partially degraded or entangled with ribosomes and thereby spread throughout the sedimentation diagram. In this event the postulated properties will be difficult to observe.

In summary, the hypothesis that nascent R-RNA associated with neosomes forms the unit responsible for the synthesis of ribosomal protein leads to a number of self-consistent relationships. The RNA of a 70S ribosome is long enough to act as template for six molecules of ribosomal protein. As the complete 70S ribosome contains 36 of these units the template must operate six times; i.e., it should pass over six points of synthesis. The cell's content of neosomes is just sufficient to provide six per template. The rate of amino acid incorporation per site is roughly the same as the rate calculated for nonribosomal proteins. The hypothesis is subject to test by observing the incorporation of tracer-labeled bases and amino acids.

L. Some Current Problems

Several of the most basic questions of molecular biology have now been answered. We no longer need to wonder whether cellular RNA is self-duplicating, whether there are many points of DNA synthesis in E. coli, or whether the information for the assembly of proteins comes from the ribosome. Yet these were all important questions only a few years ago. The qualitative description of precursors, products, and templates is reasonably complete.

The few discordant facts that remain seem more likely to provide additional details to the present theories than to upset them. For example, the theory that S-RNA carries amino acids to the protein-forming template is widely accepted and is supported by a wealth of experimental evidence derived mainly from in vitro experiments. In living cells, however, the S-RNA-amino acid complex does not show the kinetics expected of an obligatory intermediate in protein synthesis (IV.C.7). To reconcile this fact with accepted theory we must postulate that only a very small fraction of the cell's supply of S-RNA is active as a carrier. If most of the S-RNA is held out of the reaction, the concentration of the active form throughout the cell would be so low that diffusion rates might be limiting. Alternatively, the active fraction might represent the S-RNA molecules that are held in close association with the polysome complex. In this event the diffusion rate should be adequate because the local concentration would be high. Thus a resolution of this paradox might provide additional information on the structure and function of the polysomes.

Another discordant observation is the prolonged period of protein synthesis that occurs when 5-fluorouracil is supplied to a uracil-requiring mutant (IV. B.6). During this period P^{32} does not seem to be incorporated into new template material but protein synthesis proceeds long beyond the expected lifetime of existing templates. Once again, an understanding of this effect should bring additional information about the degradation of templates in normal growth conditions.

Beyond these relatively minor difficulties lies quite a different area where present knowledge only poses the questions but offers no answers. One general question concerns the nature of the mechanisms controlling RNA synthesis. Another is about the details of the reactions that permit precision in the replication of nucleic acids. Our studies of material flow provide little additional information about this. We can only record the rapidity of the events, which are carried out with errors held to a level of 10^{-10} . The rules of complementary pairing seem to be followed, but complementary pairing in itself cannot account for the accuracy. Presumably, at the time life originated the chemical forces provided an approximate replication process which was later made more accurate by the additional specificity of enzyme action.

Studies of the cellular materials and their kinetics can, however, contribute some data on control mechanisms. At least, they can help to frame the questions sharply and can place some limitations on the possible answers. The remaining paragraphs of our summary will attempt this task to the extent that it can be done without reviewing all the other relevant data.

Control of gene transcription. The rates of RNA synthesis vary widely, depending on the growth conditions and on the particular species of RNA. Unspecific control mechanisms such as the supply of nucleotides or quantity of polymerase are adequate to explain the limitations on maximum rates and variations from one medium to another. The cessation of RNA synthesis caused by lack of an amino acid has been attributed to inhibition of the polymerase by uncharged S-RNA.²⁶ These mechanisms, however, are inadequate to explain the variation in the rates of transcription from one gene to another and the variations caused at one gene by an inducer.

Such individual variations may be attributed to the state of the DNA that acts as template. A few genes operate at high rates; others at variable rates, depending on the presence of small molecules that act as inducers or repressors. It is commonly assumed, therefore, that all genes have the capacity to operate at maximum rates and would do so unless repressed by some external circumstance. Since most genes are producing very few RNA copies it appears that most genes are repressed. The repression is usually attributed to the specific repression of a large number of individual sites. Alternatively there might be a general repression which can be counteracted at individual sites. In either event there should be molecules that can recognize a particular location

along the DNA and can act at that point either to repress or to relieve a general repression.

The number of these control regions is probably considerably less than the number of genes. Frequently a number of genes are expressed (or repressed) together,²⁷ and the gene products found in polysomes seem larger than the product of a single gene.²⁸ Assuming that the genes are controlled in groups of five, *E. coli* would require 1200 control regions.

The requirement for 1200 control points follows from the assumption that all gene groups are equivalent and all would be transcribed at equal high rates unless repressed by some external circumstance. This view is based on observations of the behavior of a limited number of genes, a few hundred at most of the possible 6000 in *E. coli*. It is by no means certain, however, that all genes can produce RNA copies at the maximum rate. Perhaps the large majority have not been the subject of intensive study simply because they cannot be induced and never produce more than a few RNA copies. If each gene group has its initiation point marked by a distinctive sequence of nucleotides, the same sequence or an adjacent one could also carry information designating the frequency of reading. One sequence might indicate a high probability of transcription, as for example in the genes specifying the ribosomal RNA and S-RNA. Another sequence might indicate the very low frequency of reading that seems to be the typical situation. A third class could indicate the variable frequency of reading so familiar from studies of induction and repression. The mechanism for conveying this information could be simply a variation in the affinity for the RNA polymerase.

The marking of the initiation point seems to be a logical necessity; otherwise, most of the RNA synthesis would be initiated at the wrong point. Hence it seems quite possible that the rate is also indicated. If this is indeed so the number of points requiring external control would be considerably less, corresponding only to genes that are affected by specific inducers or repressors. Accordingly, control material would be present in much smaller quantities and would be more difficult to identify.

If most genes carry their own regulators as part of their structure the search for repressor molecules will be almost hopeless. It therefore seems more profitable to return to the assumption that some 1200 regions must be repressed. The logical basis for this procedure is similar to that of the man who looked for a lost key under a street lamp. If it were in any other place he would not be able to see it.

General or specific repression. The characteristics of repressor material should be quite different for the alternative types of repression. The concept of specific repression is well known and needs little discussion. General

repression has had relatively little emphasis, and some consideration is needed to visualize mechanisms compatible with facts now known.

The structure assumed by the DNA in the confined space of the nucleus could result in a general repression of gene expression. Rotation of the DNA around its axis is expected to result from the unwinding that occurs on replication. It is possible that the entire DNA of the genome rotates as a unit (at speeds as high as 500 rps), but it is also possible that there are points of free rotation which act as swivels along the length of the DNA. If so, different regions of the DNA would be free to rotate or not, depending on interactions with other cellular elements.

The rotation of the DNA could also play an important role in RNA transcription. Either the DNA must rotate, or the polymerase and its product RNA strand must circulate around the stationary DNA. In the second model two large molecules would need to make 100 orbits per second through the closely packed, viscous material of the nucleus. In the first model the RNA product could peel off like the shaving from a lathe. If, in fact, rotation of the DNA is an important factor in RNA transcription, a general repression could be exerted simply by impeding the freedom of the DNA to rotate. When DNA synthesis occurs, the region between the point of replication and the next (assumed) swivel must rotate. In this region RNA synthesis could occur, thereby accounting for the minimum rate of RNA transcription. The general repression could be relieved by allowing free rotation of limited regions of the DNA. Unfortunately for this hypothesis, there is no evidence for the required multiplicity of swivels.

Basic proteins provide a less fanciful way of exerting a general repression. Their affinity for nucleic acid is well known, and they could simply cover the regions where RNA synthesis is initiated or hold the DNA in a particular structure. Histones have been shown to inhibit DNA-dependent RNA synthesis *in vitro*.²⁹ The repression could be relieved by displacing these proteins from specific regions or by allowing a part of the DNA to assume a configuration suitable for transcription.

The role of the control genes is not immediately apparent in terms of general repression. The control genes should determine whether or not the general repression affects the RNA output of various other genes, perhaps by determining the nuclear structure or perhaps by producing various kinds and quantities of basic proteins. In terms of specific repression, the control genes are the sources of the repressors that regulate the RNA output. One conceptual difficulty lies in visualizing a hierarchy of secondary control genes that repress the output of the primary set of control genes. A mixed model in which control genes are postulated to be self-repressed or to lie in a generally repressed region of the genome may avoid the logical difficulties that arise in either pure model.

The concept of specific repression is generally favored, but a few experiments are easier to interpret in terms of general repression. Cells growing with a very severely limited carbon supply produce the maximum proportion of β -galactosidase without inducers present.³⁰ Cells cannot be induced immediately after a period of DNA synthesis without concurrent protein or RNA synthesis (IV.C.8). Genes carried by lysogenic phage are less actively expressed after incorporation into the host genome.³¹ In fact, the experiments usually cited to indicate specific repression³² can be interpreted as indicating that a general repression is established when the newly incorporated DNA is integrated into a definite structure.

Cells have a habit of being considerably more complicated than the models designed to interpret their behavior. It seems quite possible that both specific and general repression (self-regulation) contribute to the control system. More information is needed before any of these concepts should be discarded.

Control material. Whether repression is general or specific, there must be some material in the cell capable of acting at specific regions along the DNA. One possibility is that one stretch of DNA acts to control another stretch which it recognizes by a homology in its nucleotide sequence. In this event there would be no material whose sole function was to act as control, but this type of interaction might place impossible constraints on the structure of the DNA as it occurs in the nucleus. It is therefore necessary to inquire whether the constraints already present are compatible with such an additional requirement.

The first requirement on the structure of the nucleus is that the DNA be coiled tightly enough to fit into the available space. Second, the coiling must allow rotation during synthesis and separation of the product strands. Finally, RNA products must be made concurrently and allowed to escape from the nucleus. For simplicity let us assume that the diameter of the coils is the maximum allowed by the dimensions of the nucleus of E. coli, roughly 5000 A. As the total length of the DNA is about 2×10^7 A, 1300 turns would be needed, each turn containing the DNA of about five genes.

In such a structure, control by interactions between adjacent turns seems possible except at the point of replication. Interactions between rotating strands are difficult to accept, and so this model seems to require numerous points of free rotation in the DNA strands.

RNA seems to be a far more likely ingredient of the control material. If the region to be controlled is to be recognized by the sequence of nucleotides in the DNA it should extend over at least 12 nucleotide pairs. Roughly 6 occurrences of any designated sequence of 10 nucleotides would be expected in a random polymer having the length of the E. coli genome. If, however, an RNA molecule includes 12 or more nucleotides it should have sufficient specificity to

form complementary pairs with only one region of the DNA. The quantity of material needed would be very small. The number of control points may be as low as 1200, and so the minimum quantity of RNA could be 14,400 nucleotides, less than 2 per cent of the D-RNA.

Control material should have a dual function. In addition to interacting with DNA it should show specificity toward the small molecules that act as inducers. RNA seems to be ideally suited for the first of these requirements but to be entirely lacking in any relationship to the small molecules. Pure RNA by itself does not seem to be sufficient.

Proteins are noted for their versatility; perhaps they could play both the needed roles. At first sight this possibility is extremely attractive because enzymes are known that act on specific regions of RNA, presumably selecting the correct region by recognizing a sequence of nucleotides.³³ In this situation the specificity may be far less, requiring interactions with only a few nucleotides. To interact with 12 nucleotides the protein would have to extend over a region 40 Å long including a full turn of the DNA helix. This would seem to be an over-extrapolation of the ability of enzymes to recognize short sequences.

Although protein does not seem to be a suitable molecule to recognize sequences in the DNA it does seem a necessary ingredient of the control material for the recognition of small inducer molecules. As these commonly have a structural resemblance to the substrate of the product enzyme, the control material would be expected to carry a structure similar to the active site of the enzyme. It is difficult to see how this property can reasonably be attributed to pure DNA or RNA; some protein must be included. If 1200 different species are needed, one-fifth of the genes would be occupied in making control proteins. Furthermore, if these genes are copied once per generation and each template produces 50 molecules of protein of molecular weight 31,000, then 3 per cent of the protein would be in this class.

Properties of both protein and RNA are desirable in the control material; accordingly, a nucleoprotein seems to be needed. The characteristics, however, are quite unlike any of the usual nucleoproteins. To control the formation of a particular enzyme, the RNA moiety of the control material might include a part of the template for that enzyme and the protein moiety might include the active site of the enzyme. Such a combination is not known in the cell. Once the protein is formed and leaves the ribosome it no longer has any relationship to the template RNA.

In one particular circumstance protein might remain associated with its template. Zipser has observed that a very small part of nascent protein remains permanently attached to ribosomes, evidently by some failure of the release mechanism.³⁴ If the same ribosome also carried a small bit of the template it would comprise the postulated but unlikely combination.

Such particles accumulate in induced cells and would be expected to influence the kinetics of induction if they were the actual repressors. Moreover, such a mechanism would not provide for any influence of the control gene. Following this line of thought, it seems possible that some similar particles might be the product of the control genes themselves. Possibly the control genes have sequences in common with the genes they control and the RNA produced by them can produce a protein with an active site. Alterations in the protein structure could cause failure to release from the ribosomes. Such a complex would combine the three properties needed for control material: a relationship to the sequence of nucleotides in the DNA, a relationship to the substrate of the controlled enzyme, and a dependence on control genes. Thus it should be worth while to search for a new class of RNA having the following characteristics: (1) small size, 12 to 90 nucleotides; (2) probably DNA-like composition, but certainly the ability to form a hybrid duplex with DNA; (3) a much longer lifetime in the cell than other D-RNA; (4) tight bond to ribosomes. These characteristics are sufficiently distinctive that the isolation and identification should be possible if the material exists. In any event, the postulated control material, whether a repressor or an activator, should be present in sufficient quantities to be detected. The repressor has had a name for a long time; it needs a body.

My colleagues E. T. Bolton, R. J. Britten, D. B. Cowie, and B. J. McCarthy have supplied most of the data on which the rate calculations are based. They concur in the calculations, but they should not be held responsible for the speculations set forth in this or the preceding section. Richard B. Roberts.

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VII. BRIEF HISTORICAL SKETCH

A. The Good Old Days

"Once upon a time" the Department of Terrestrial Magnetism confined its interests to terrestrial magnetism (1904 to 1912). By 1924 the magnetic survey situation had changed, and the most attractive magnetic studies were concerned with the extraterrestrial component. After observing variations that seemed to be due to circulating currents in the ionosphere, Gregory Breit persuaded Merle Tuve to join the Department and probe for the currents with radio pulses. By 1926, pulse techniques not only had measured the height of the conducting layer in the high atmosphere but had incidentally provided the experimental basis for pulse radar.

Wanting a more intimate contact with the ionosphere, Tuve and Breit undertook (1927-1928) to persuade President Merriam of the Carnegie Institution of Washington to contribute support to the rocket development work of Robert Goddard. They themselves turned to a study of magnetism in atomic nuclei, as this seemed a more fundamental problem and nuclei are much easier than the earth to take into the laboratory. Thus the high-voltage accelerator and nuclear physics program at the Department was initiated (1927).

The biophysics program was conceived shortly thereafter, when Merle Tuve married Winifred Whitman, M.D. The initial experiments included studies of radio-frequency heating in animals placed near the high-power radio transmitter used in the ionosphere work. New types of health hazards were visualized for workers using high-energy accelerators, and they were explored by exposing rats to high-energy gamma rays.^{1, 2}

In the years following 1932 nuclear physics equipment was in use at the Department for measurements of the short-range forces that bind the atomic nucleus. It also produced high-energy radiations and artificial radioactivity and was therefore an object of great interest to biologists. Among those requesting cooperation were M. Demerec, who wanted his fruit flies bombarded with high-speed protons; P. Henshaw, who asked for equal time for his frog eggs; L. B. Flexner, who used Na^{24} to measure transfer from mother to embryo; J. H. C. Smith, who, with Dean Cowie, observed early stages in photosynthesis using C^{11} , despite the limitations arising from its 21-minute half-life; and E. Friedheim, who needed As^* to measure the incorporation of arsenical drugs by the trypanosomes of infected rats.

By 1939 the demands of biology had become heavy. L. R. Hafstad noted that the log of the newly completed pressure Van de Graaff generator showed roughly half-time for biologists and half-time for repairs.

To handle the growing needs of the biologists, Tuve arranged to have D. B. Cowie appointed as Fellow of the National Cancer Institute for the specific purpose of taking care of the biologists in their work at the Department. Cowie's enthusiastic participation resulted in greater success with the tracer experiments and increased needs for machine time.

When Vannevar Bush was elected President of the Carnegie Institution of Washington in 1938 he initiated discussions with Tuve which led to the decision that the Department of Terrestrial Magnetism should have a cyclotron and laboratory to provide for the needs of biologists of the Institution and the Washington and Baltimore areas. It was hoped that the cyclotron would have sufficient capacity to produce tracers in abundance and yet leave time for physics experiments.

In those days cyclotrons could not be ordered from the catalog, and R. B. Roberts (Carnegie Institution of Washington Fellow 1937-1939) was appointed to the staff to assist in designing and building the apparatus. After receiving their doctorates under Professor E. O. Lawrence, who was a boyhood playmate and friend of Tuve in South Dakota, P. H. Abelson and G. K. Green were brought from the University of California, Berkeley, to join the project.

By the fall of 1940 the design and purchasing for the cyclotron were well along and parts were being delivered. Abelson, Green, and Roberts became occupied with various war activities, and work on the cyclotron slowed and stopped. It was needed, however, possibly for work on nuclear fission and certainly for tracer production for medical research. The situation was rescued by the assignment of Cowie to this wartime project, and the cyclotron was completed under his direction in 1943. He was transferred from National Institutes of Health to the CIW staff in 1944 to use the apparatus for urgent medical problems being encountered in various theaters of the war. It was used intensively to produce tracers for studies of tropical diseases by a group including Cowie, Brady, Lawton, Ness, Endicott, and others at NIH and the Navy Medical Center.

From these prewar and wartime experiences the CIW physicists learned that biological experiments could be exciting and rewarding if they shared fully in the planning and execution of the experiments, but that merely performing the needed bombardments and measuring samples were uninspiring chores.

B. The Beginnings of the Biophysics Section

When the Carnegie Institution of Washington resumed normal activities after the conclusion of the war, the program of the Department of Terrestrial Magnetism was drastically revised. Tuve, who became the new Director, was an experimental physicist, in contrast to the former Director, John A. Fleming, whose experience had been in observations. Furthermore, Tuve had experienced large-scale wartime operations. He realized that nuclear physics had acquired

such practical importance that it was bound to receive the highest level of support. The modest contribution the Carnegie Institution of Washington could make in this area would be overshadowed. Nuclear physics did not seem attractive as a major effort to be supported by endowment funds of the Institution.

In biophysics, however, the use of tracers, which had just begun, clearly opened opportunities for answering questions that had previously been unapproachable, and Tuve chose this area for emphasis. Cowie and his associates were already engaged in this type of work. On Abelson's return from his atomic-power work for the Navy, a new Section of Biophysics was formed, with Abelson as chairman.

W. R. Duryee, who returned from active duty in the Army to join the NIH staff, was allocated room for a cooperative program. The initial experimental work centered around Cowie's wartime projects and the application of tracer techniques to the field of Duryee's interests. At the same time, a number of biologists were invited to hold sessions on varied topics of active interest. The Washington Conference on Theoretical Physics that year was devoted to biology, and its participants were equally divided between physics and biology.* But one of the visiting biologists described the DTMBiophysics Section to his colleagues as "A group of wistful physicists contemplating the mysteries of biology." From this period of contemplation, however, emerged the conviction that macromolecules and the patterns and pathways of their syntheses posed the most significant problems, a credo that has determined the objectives of the group for sixteen years.

Roberts, who had participated in the postwar biophysics planning for the Department, also returned from wartime work in early 1947, but he was occupied with nuclear physics. In fact, he even deflected Abelson from biophysics long enough to collaborate in a study of the angular distribution of neutrons.³ One day in July, however, the prospect of three weeks at a place called Cold Spring Harbor offered an alluring contrast to the Washington heat. After graduating from the phage course and taking three weeks' postgraduate work with

*The Ninth Washington Conference on Theoretical Physics was held from October 31 to November 2, 1946. Its topic was "The Physics of Living Matter." Those attending from outside of Washington were:

G. W. Beadle	J. Franck	F. O. Schmitt
J. W. Beams	S. Karrer	S. Spiegelman
N. Bohr	W. J. Kautzman	W. M. Stanley
C. F. Cori	J. G. Kirkwood	L. Szilard
M. Delbruck	F. W. London	E. Teller
M. Demerec	H. J. Muller	J. von Neumann
J. T. Edsall	E. O. Salant	H. Weyl

H. B. Newcombe at the Institution's Department of Genetics, Roberts returned thoroughly infected with enthusiasm for bacteria and viruses. This contamination soon spread throughout the laboratory.

In 1948, Hugh H. Darby joined the Biophysics Section as special Fellow of the Carnegie Institution of Washington. Both Darby and Duryee brought a wealth of biological experience that was invaluable to the physicists. Close association with these men, who were enthusiastic teachers, was the equivalent of years of formal training.

Ellis T. Bolton joined the Section in 1949 as a Carnegie Institution Fellow, and Roy J. Britten came in 1951. In 1953, Abelson left the group to be the Director of the Geophysical Laboratory of the Institution and Roberts became Chairman. Brian J. McCarthy joined the Section as a Fellow in 1958. In 1962 Bolton took over the duties of the Chairman.

In addition to those mentioned above who formed a permanent nucleus the Section has enjoyed a continuous flow of Fellows, visiting investigators, and research associates. Those who spent more than three weeks with the group are listed in Appendix 1.

C. The Formulation of a Program

Concurrent with the growth of the Biophysics Section to its present size of five staff members was the development of concepts and techniques. The original basis for building a cyclotron was stated in Year Books 38 and 39 as follows:

NUCLEAR PHYSICS AS A TOOL FOR BIOLOGY AND CHEMISTRY

It is obviously impossible for a specialist in one field of research to have a working knowledge of all the recent developments in other fields of research which may have a useful bearing on his own problems. On the other hand, fundamental work in any scientific field, however specific its own objective may be, has a way of embracing in its significance and its applications many other scientific interests. The Department's work in nuclear physics is an example of this which promises to be of special importance in particular fields of the Institution's activities. Started in 1926 as an attack on the fundamental problems in magnetism, this work in recent

years has developed unexpected potentialities as a tool for investigating certain important chemical and biological problems. Radiations from nuclear-physics apparatus have lethal and genetic effects somewhat different from those previously available. This provides new analytical possibilities which are being developed in the Department's laboratory in cooperation with the Institution's Department of Genetics and by Dr. P. S. Henshaw of the National Cancer Institute. Another procedure, of wider usefulness, is that involving the use of radioactive isotopes (species) of the ordinary chemical elements as "tracers" in quantitatively following a given sample of one element through even the most complicated and unknown chemical

reactions, in spite of the initial presence of the same element in large amounts. Radioactive isotopes of nearly every chemical element can be produced by the use of nuclear-physics equipment and procedures. During the past two years several programs of cooperative research using radioactive tracers have been put into operation, notably in Copenhagen, in Berkeley, and in Rochester, and have yielded new information on the physical chemistry and particularly on the biochemistry (physiology) of sodium, phosphorus, iron, and iodine.

A limited attempt to explore the value of this technique was made by the Department's staff in cooperative studies of three problems. With Dr. L. B. Flexner of the Johns Hopkins Medical School, measurements have been made on the rate and amounts of transfer of radioactive sodium across the placental boundary, using pregnant rats, as an initial approach to problems concerning

the nutrition and development of the fetus. With Dr. Keith Brewer of the U. S. Department of Agriculture, studies of plant-nutrition are in progress, using radioactive sodium, potassium, and phosphorus. Another project of special interest is the use, in the Department's laboratory, of radioactive carbon by Dr. J. H. C. Smith of the Institution's Division of Plant Biology for studies of the way in which plants take up carbon dioxide in the dark, whether by simple solubility or by the formation of temporary compounds, as a fresh attack on the many broad and unsolved problems of photosynthesis.

Plans are being developed for the construction of special facilities, including a large cyclotron, to extend this type of attack to many biological and chemical problems within the Institution and in the many other research laboratories in and near Washington.

And from Year Book 39:

THE CYCLOTRON PROGRAM

An expansion of the Department's activities in nuclear physics to embrace an enlarged program of fundamental work in biology and chemistry, utilizing the artificially radioactive isotopes of ordinary elements as tracers for following various reactions, was undertaken. This program will center around a large cyclotron, essentially a duplicate of the 60-inch cyclotron installed last year at Berkeley, and will involve cooperation with various members and groups of the Institution's staff working in chemistry and biology, and with a number of other research organizations in the Washington area, such as the National Cancer Institute, the Department of Agriculture and other federal agencies, one or more of the local universities, and the Johns Hopkins University in Baltimore. In addition to the work with radioactive tracers, the cyclotron will provide for still further exten-

sion of the Department's studies of atomic nuclei and the primary particles of matter.

For putting this large project into operation, the first necessities were the selection of personnel, the design and construction of a highly specialized laboratory building, and the construction and installation of the cyclotron itself. Roberts, Abelson, and Green were selected to work with Tuve on this project, and Cowie was assigned here from the staff of the National Cancer Institute. The new laboratory is well under way, and many of the large parts of the cyclotron are completed or under contract for early delivery. It is expected that the remaining details of small parts, wiring, installation of controls, and assembly may be completed by July 1941.

An important part of the initiation of this project, which contemplates the joint activity and initiative of investigators in widely separated fields for the broadly defined purpose of fundamental research, aside from thera-

peutic questions, is the development of a sound basis for guiding the work with regard both to technical and to administrative or liaison questions. Actual experience in such cooperative work is the only reasonable basis for guiding the project as a whole, and arrangements were made to supplement our previous experience by pushing forward a group of specific problems, using the existing high-voltage equipment, concurrently with the construction of the cyclotron. These efforts already have been valuable in a number of ways, even including modification of the building plans to incorporate features peculiar to the requirements of simultaneous tracer-work with different radioactive elements, recognized only as a result of operating two such projects simultaneously. The gaining of experience on a small scale before attempting to utilize the great output from a 60-inch cyclotron has been valuable to the cooperating agencies as well.

SHIELDING

The powerful radiations from a cyclotron are dangerous for personnel of the laboratory unless arrangements for adequate shielding are provided. In addition, much smaller amounts of stray radiation or activity in the rooms of the laboratory will give a large and fluctuating background on the instruments used for following radioactive tracer-samples. The shielding arrangements necessary for a specified reduction of intensity were not known, but calculations made on the basis of measurements using the Department's high-voltage equipment, checked by rough measurements on existing cyclotron-installations, indicated the necessity for completely enclosing the 60-inch cyclotron, and providing an 8-foot cover of moist earth overhead to prevent back-scattered radiation from the air above the laboratory. Numerous technical features were incorporated in the building to reduce the importance of stray radiations, including a separate instrument-room, shielded over all by an additional 3 feet of earth, for measurements at high sensitivity. The shielding for personnel was based on hospital experience with X-rays, but an added safety-factor of 10 was used because of the still unknown risks of radiation-exposure. Study of the

shielding question showed that, although geneticists emphasize the cumulative hereditary effect of radiation-exposure, no genetic experiments using other than massive single doses of radiation have been performed, even with *Drosophila*. Arrangements were accordingly made for a preliminary test of the genetic effects of continuous radiation-exposure (20 roentgens per day) over six or seven generations of *Drosophila*, the experiments being made jointly by the National Cancer Institute and Dr. Demerec of the Institution's Department of Genetics. Cowie stimulated this investigation and also arranged for the Cancer Institute to undertake a survey of the actual exposure to radiation (X-rays and gamma rays) of the personnel in various near-by hospitals and clinics. About half of these groups exceeded the customary tolerance-dosage of 0.1 roentgen per day. This survey is being extended to all parts of the United States.

COOPERATION IN BIOLOGY AND CHEMISTRY

Photosynthesis. Cooperative experiments over several months were made at the Department with the Institution's Division of Plant Biology, using radioactive carbon (C^{14}) for studies of the various ways in which plants take up carbon dioxide for use in photosynthesis. A plant has a reservoir-mechanism for carbon dioxide taken in from the surrounding air. The absorption of CO_2 into living sunflower leaves by solution in the water of the sap, by reaction with insoluble carbonates, and by reaction with the soluble buffer-substances were processes found to be in operation. In addition, CO_2 reacts to form a non-carbonate derivative of which little is yet known. It has been found that the active absorption of CO_2 is not a necessary part of the initial photochemical reaction, since CO_2 absorbed before illumination can be used for the process of photosynthesis. Whether the carbon newly assimilated in photosynthesis is lost by respiration more rapidly than the carbon from other organic compounds already contained in the leaf has not been determined, but it was shown that this loss due to respiration is rapid.

Arsenic. Another project in this initial program of cooperation in biology and chemistry was the study of the distribution of radioactive arsenic in animal tissues in cooperation with Dr. Ernst A. H. Friedheim, of the University of Geneva. Friedheim, Abelson, and Cowie used radioactive arsenic for the synthesis of a pair of arsonic acids, one of high chemotherapeutical activity (arsanilic acid), and the other of no chemotherapeutical activity (p-arsonobenzoic acid). The distribution of the arsenic of these compounds in various organs of rats, guinea pigs, and rabbits was studied *in vitro* and *in vivo*, the arsenic concentration being determined quantitatively by measurements of radioactivity. Both compounds had similar distribution in animal organs with the exception of the blood. Both showed a definite accumulation of arsenic in the kidney, liver, and skin, as compared with the blood. The concentration in the brain, however, was only a fraction of that in the blood. It was also found that the red blood cells of the rat concentrated the arsenic of the chemotherapeutic arsanilic acid *in vivo* and to a lesser extent *in vitro*, whereas the arsenic of the inactive p-arsonobenzoic acid was not concentrated in these cells. This finding will be highly significant if it proves to be generally true of chemotherapeutic compounds. The concentration of arsanilic acid in the blood was determined as a function of time and mode of administration.

Placental permeability. Drs. Louis B. Flexner and H. A. Pohl, of the Department of Anatomy, Johns Hopkins University, were assisted in an investigation using radioactive sodium (Na^{24}) to measure the transfer of sodium across the placenta in several groups of animals. Each group of animals studied had a different placental structure. The experiments with the cat have shown several interesting results. The fetus comes to within 10 per cent of a limiting equilibrium-value with respect to sodium ions in the maternal plasma after 16 hours, in striking contrast with the extracellular fluid of the mother, which comes to the same equilibrium in about 4 minutes. The rate of transfer to the placenta (per unit-weight of placenta) has been shown

to be very low in the earlier stages of pregnancy (15 to 20 days), but increases linearly to 60 times this value at 57 days. A decrease to term is then observed in this rate. The rate of transfer to the fetus, however, is high in these earlier stages and falls off with duration of pregnancy. This may be explained on the basis of the ratio of the size of the placenta to that of the fetus, this ratio decreasing as the fetus develops.

Neutron-irradiation. Dr. Demerec, of the Institution's Department of Genetics, has in the past investigated the dosage-effect relationship of 1000 to 5000 roentgens of X-rays on *Drosophila*, both by genetic and by cytological methods. To determine the comparative effects with neutron-irradiation, *Drosophila* of the same strain used for the X-ray investigations were given 2000 "roentgens" of neutron exposure and sent to Demerec for analysis. One interesting point being examined is whether the intense local ionization due to neutron-recoils may produce a higher frequency of multiple chromosomal breaks than with X-rays. Further work will be undertaken with the higher neutron-yields of the new cyclotron.

Neutron-bombardments of tissue-cultures were made for Dr. George O. Gey, of the Johns Hopkins University. Measurements made on the radiation received by his control tissue-cultures revealed that these controls accidentally received from 40 to 100 times the residual irradiation normally arising from radioactivity of surroundings, owing to the proximity of radium sources. One of these control-cultures of normal rat tissue underwent a spontaneous transformation and acquired tumor-producing characteristics, proved by injection and growth. This malignant transformation is of special interest in connection with the question of the effects of long-continued irradiation.

Plant-nutrition. Radioactive sodium and phosphorus samples were supplied to Dr. Keith Brewer, of the U. S. Department of Agriculture, for certain studies in plant-nutrition. He measured the absorption and elimination of these elements by plant-roots in relation to temperature, pH, antagonistic ions, and other factors.

These paragraphs, written before the war, show a clear appreciation of the value of radioactive tracers for biology and indicate areas of our local experience in a range of biological problems. It was not foreseen at the time (1) that tracer molecules would become more important than tracer atoms; (2) that the biologists would soon lose their dependence on physicists for the production and measurement of radioisotopes; (3) that the reactor would supplant the cyclotron as a source of isotopes; (4) that physicists had more than technique to contribute to biology.

The early reports of the Biophysics Section continued to emphasize co-operative projects. The physicists could contribute their experience with tracer technology but did not feel ready to select significant biological problems. We may quote a paragraph from Year Book 46, written in the early months of 1947:

With the production of isotopes, both stable and radioactive, biology and general physiology have a new approach to many of their unsolved problems. The technique and the philosophy implied by these new physical tools necessitate a knowledge of and respect for two sets of training—that of the physicist and that of the biologist—before a good biophysical approach can be

made. The accuracy of measurement and the micro-quantities capable of use both lead to new forms of instrumentation and permit a deeper probing of the life processes than was possible heretofore. The selection of the problems and of the particular animals or plants to be used has to be based on a wide knowledge of general biology.

Later in the same report (p. 76) we find:

Long-range biophysics program. To date biophysics has little recognized standing as a separate scientific discipline. Nevertheless, there is a widespread conviction that the processes in living matter involve important physical phenomena. How can physics best make its contributions to answering crucial questions concerning living matter? To date this question has not been answered. The efforts of our group of several physicists interested in

biological problems will be directed during the next several years toward the formulation of questions in this field which can be regarded as fundamental to living systems and yet can be made accessible to attack and observation in terms of physical processes. Most of the present specific problems lie squarely across the three fields of physics, chemistry, and biology. Close collaboration with certain workers in each field is necessary and planned.

The reports issued during the next two years, 1948 and 1949, continue to emphasize the educational process needed by the physicists so recently converted to biology. During this period the publications reflect the interests of the collaborating biologists.

The first indication of any self-confidence appears in the following passage from Year Book 49, written in mid 1950:

Special provision was made by the Institution in 1938 for a cyclotron, a new laboratory building, and a small group of physicists at the Department in order to underwrite a fresh and vigorous collaboration with the various medical and biological investigators in the Washington-Baltimore area. As a natural extension of the Department's work in nuclear physics, the use of radioactive tracer isotopes and other techniques and ideas of modern physics for studies of living matter offered new challenges and opportunities. The postwar availability of radioactive isotopes from Oak Ridge has reduced the service load on the group and has permitted a

vigorous program of individual research on biological material by these physicists. This program is beginning to bear fruit in substantial research contributions, despite the long preparation necessary before a man can be creative in the areas where physics, chemistry, and biology merge. It is natural that most of the experimental work is biochemical in nature, but the actual measurements carried out are not made primarily for their usefulness in application, but as further stimulus for the investigation of process and order in living matter, as viewed by physicists from their own specialized background of quantitative training and experience.

This was followed by a declaration of independence in the introduction to the Biophysics Section report in Year Book 50, written in mid 1951:

One of the new lines of endeavor initiated when the Department resumed its research activities at the close of the war was a program in "biophysics"—quantitative research in biology carried out by investigators trained in physics. This characterization of biophysics was reached after experience with several other ways in which the developments of modern physics contribute to the understanding of biological processes. The development of new instruments as an end in itself was rejected because such an endeavor would have permitted only superficial contact with biology. Measurement of the physical properties of biological material is relatively unattractive unless required in the study of a particular biological problem. Finally, a restriction to the study of the interactions of living organisms with physical agents seems to be exceedingly narrow in significance. Though these other possible lines of effort would be almost certain to produce useful results, it appeared that the

application to biological problems of training and experience in physics, and not only the techniques of physics, might be of more value in the long run.

This point of view regarding biophysics was the outgrowth of several years of experience in collaborating with biologists on biological problems which required radiation or radioactive isotopes. Before 1946 both tracer materials and the equipment for measuring radioactivity were generally unavailable and unfamiliar to the biologists. Experimental programs utilizing tracers were undertaken as a co-operative venture, with both physicists and biologists participating. These collaborative efforts showed that the physicist had to become familiar with the biological problems if he wished to make contributions of ideas as well as techniques. Furthermore, it was apparent that the physicist was quite different from the classically trained biologist both in his way of thinking and in his approach to the problems of biology. In the hope that these differences might lead

to valuable contributions, a small group of physicists was invited to investigate biological problems.

During the four years that this program has been under way the area of interest has become well defined. This area includes the processes by which a single cell organizes the material and energy from its surroundings into a duplication of itself, and the processes by which the cell reorganizes its functions to meet changes in the environment. It excludes the complicated interactions between different types of cell which are so important to the study of higher organisms. The material used in our program covers a wide range of unicellular organisms including bacteria, bacteriophage, yeasts, actinomyces, and molds.

For most problems, however, *Escherichia coli* has proved to be very useful. One major advantage of this organism lies in the permeability of its membrane to most ions and molecules; the membrane seems to obstruct the free passage of only the giant molecules of proteins and nucleic acid. Consequently the organism is almost completely exposed to its environment, and the effects of changes in the environment are quickly apparent.

The problems of immediate interest are those related to protein synthesis, nucleic acid synthesis, the function of nucleic acid, and its relation to protein synthesis. These processes as they occur in the normal growing cell are studied by various methods. A single experiment may involve the use of the Warburg respirometer, the spectrophotometer, ion-exchange columns, paper chromatography, and particularly radioactive tracer-labeled compounds.

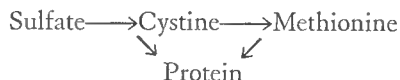
During the past year the pathways for synthesis of many of the amino acids (the building blocks of proteins) have been examined by following the incorporation of carbon dioxide and sulfur from the medium. Studies of CO_2 and PO_4 metabolism have given new information on nu-

cleic acid synthesis. The investigation of the metabolism of a deficient mutant throws some light on the relation between nucleic acid synthesis and protein synthesis. Finally, observations of the effects of manganese on cells offer a new approach to the study of the function of nucleic acid.

One particular method, the competitive utilization within the cell of labeled compounds supplied in the nutritive mediums (usually intermediate products of partial synthesis), has been used more and more frequently during the past three years and has proved to be exceedingly valuable. It appears that the usefulness and versatility of the method are not generally recognized, as it is used only occasionally in other laboratories.

The method is best explained by considering as an example its application to the study of sulfur metabolism. *Escherichia coli* can utilize sulfate ions as the sole source of sulfur. Chromatographic analysis of the cells shows that the sulfate is used mainly in the synthesis of the sulfur-containing amino acids cystine and methionine. If the cells are grown in the presence of tracer-labeled sulfate, the tracer is found in both cystine and methionine. If radioactive cystine is utilized as a sulfur source, both the cystine and the methionine are again radioactive. If, however, the cells are grown in the presence of radioactive sulfate together with stable cystine, no radioactivity is found in the cell. This shows that although sulfate is adequate to support growth, it does not compete with the preferred sulfur source, cystine. Two factors operate in this competition: (1) Further synthetic processes using cystine as a raw material utilize the abundant cystine available in the medium rather than the cystine which is synthesized biologically; (2) the presence of exogenous cystine shifts the equilibrium of the synthetic reactions which produce cystine, and the quantity of cystine synthesized by the cell is reduced.

If methionine and sulfate are together in the medium, it is found that the methionine of the cells is derived from the methionine of the medium, whereas the cystine of the cells originates from the sulfate of the medium. These results show that the general pathway of sulfur metabolism is as follows:



This pattern had already been suggested by the growth requirements of a series of deficient mutants, and it is gratifying that these independent methods lead to the same conclusion. The competitive method appears to be of more general application and less ambiguous than the use of deficient mutants, which has proved such a

powerful tool in biochemistry. In many cases the needed mutants have not been isolated, and it is often difficult to determine the changes in the enzyme system which produce the deficiency. Furthermore, the deficient mutant may have complicated side reactions not found in the normal cells. Like any other new tool, the competitive method is most valuable when used to augment rather than replace the older methods. It has already been used to advantage in the study of the metabolism of deficient mutants, showing, for example, that in a mutant requiring methionine for growth, the synthetic processes leading to cystine are operating normally. Other examples of the use of the competitive method will be found in the detailed experimental results reported below.

Contributing strongly to the development of independent formulation of problems was the increasing focus on microorganisms, particularly E. coli. Introduced into the laboratory in the fall of 1947 microorganisms diverted Cowie from his interests in capillary permeability to the permeability of E. coli. Abelson observed P^{32} incorporation in Arbacia eggs and then in E. coli. By 1950 all but one of the publications referred to E. coli.

The program, however, was still scattered and exploratory. It was still dominated by the tools and techniques available. Experiments were likely to be concerned with the permeability, concentration, and the effect of various ions. An increasing degree of sophistication was developing in measuring the rates of incorporation of tracers into various chemical fractions of the cell.

Radiation was one of the techniques explored during this period. Frequently physicists entering biology cling to some area of their previous competence. Tracer technology is one; radiation is another. Our experience with radiation left us with the general impression that it was difficult enough to determine the effect of any one known metabolic poison but that radiation damage was the result of many unknown poisons plus damage to the molecules of the cell. It seemed far less useful as a tool than tracers.

Another general choice was made during the early exploratory period. Cell-free systems, in particular isolated and purified enzymes, offered one method for the study of biology. This method was well established and applied by the biochemists; biophysics had little to offer. The operation of the intact cell seemed a more attractive area of research.

The report in Year Book 50 written in June 1951 marked the end of the exploratory period. In the years since 1947 the Biophysics Section had begun by collaborating with a number of biologists in areas of their interest. Gradually interest had centered on problems of single cells which could best be investigated using microorganisms. Diverse interests had concentrated down to a minor interest in permeability of bacteria and a major interest in the biosynthesis of small molecules.

D. Permeability

One of the simplest applications of radioactive tracers is the measurement of permeability. A common lecture demonstration during the middle 1930's consisted of drinking a solution of Na^{24} while holding a Geiger counter. Within a few minutes the Na^{24} had passed through the capillaries of the stomach and was carried by the blood to the vicinity of the counter. Thanks to an "accident" on the part of a rat, Flexner observed Na^{24} in the urine within seconds after its injection into a tail vein.

Abelson, cooperating with Duryee, measured the permeability of frog eggs and extended these measurements to Arbacia eggs. In 1938 Flexner used Na^{24} for measurements of placental permeability; these studies were continuing with Cowie's assistance in 1948.

In 1948 Cowie and Roberts combined efforts and interests to measure the permeability of E. coli to Na^{24} . For this ion the cell wall seemed freely permeable. For K^{42} , however, it behaved differently. There appeared to be a freely permeable component which equilibrated with 75 per cent of the cell volume, but there was another component that could accumulate in the cells far in excess of the surrounding concentration. The exchange of this component depended on the cellular metabolism. In view of the free permeability, this component was attributed to binding by larger molecules of the cell.

Finally, $\text{PO}_4^=$ and $\text{SO}_4^=$ showed incorporation into stable molecules in addition to penetration and concentration. Amino acids and sugars seemed to share this behavior.

Similar experiments were continued for several years. Britten's first project on arriving at the laboratory in 1951 was the investigation of the permeability of Gram-positive organisms which seemed different from E. coli.

The trend of thought was expressed in Year Book 52:

It is a common observation that the kinds and amounts of chemicals which comprise the bacterial cell remain remarkably constant in spite of radical changes in the environment in which the cell develops. Alterations in the constituents of the cell

are in general progressively less as structures become more highly organized.

Chemically, the bacterial cell may be visualized as a series of classes of chemicals, the members of the classes becoming increasingly larger and more complex

along the series. Thus, in the water space within the cell are all the constituents present in the medium. They include water, salt, sugar, and a host of other organic compounds of relatively low molecular weight. In another class are the chemicals which do not permeate freely but which may be extracted from the cells by means of cold dilute acid. These include peptides, such as glutathione, and other complex organic molecules. These compounds are part of the organized protoplasm of the cell, but their associations with proteins or nucleic acid are easily disrupted. In still another class, the alcohol-soluble chemicals, are found lipids and complex polypeptides. The polypeptide material contains the same amino acids in nearly the same proportions as the rest of the bacterial protein. Finally, there are the truly macromolecular components, the residual proteins and the nucleic acids.

The water-space components reflect every chemical change in the environment. Thus, if sodium salts in the medium are replaced by potassium analogues, potassium salts are found in the cell. Changes in the biochemical activity of the cell are also reflected by the water-space constituents, and through such changes the cell alters the environment in which it develops. For example, a medium containing glucose and homoserine supplied to growing cells will be found to contain appreciable quantities of organic compounds de-

rived from glucose, and specifically related to the metabolism of homoserine and to the synthesis of methionine and isoleucine. These compounds are not found in the absence of homoserine.

The acid-soluble portion is somewhat less variable in relation to changes in the environment. Nevertheless, it will change whenever the stresses are large enough. When *E. coli* is starved for sulfur by removal of the supply in the medium, the sulfur of glutathione is utilized for the synthesis of protein methionine and cystine, whereas normally it does not function in this way. On continued sulfur starvation, even the alcohol-soluble materials are attacked and utilized for making residual protein. Finally, all protein synthesis ceases and growth stops. In spite of these extreme conditions, the residual proteins and nucleic acids have not undergone qualitative change, and their relative proportions remain constant.

Processes which give rise to variation, as well as mechanisms which prevent change, are to be found at every level of the biochemical activities of the bacterial cell. They may be correlated with the degree of complexity of the collections of chemicals considered, and depend for detail on the particular activity studied. Whatever their specific qualities, they are orderly, integrated processes. They may be controlled, altered, and sometimes predicted.

These thoughts are elaborated in Year Book 53:

The organization of the cell poses problems which tempt speculation. Many of the features of metabolism can be interpreted in terms of a simple model of the cell, one in which metabolic intermediates diffuse freely from one active site to another within a region delimited by the cellular membrane. Such a model is helpful when metabolism is considered in a

general way. The required compounds enter the cell from the medium and undergo reactions catalyzed by enzymes. A part of the material is used to supply energy, and waste products diffuse out of the cell. Another part is successively altered by a series of enzymes to provide the building blocks for protein and nucleic acid synthesis. These low-molecular-weight

components then must diffuse from the enzymatic sites where they were formed to the multitude of different sites where the different types of proteins and nucleic acids are being assembled.

To operate efficiently according to this model, the cell would need a membrane which would allow the entry of raw materials such as glucose, ammonia, and phosphate and permit the release of waste products such as carbon dioxide. If metabolic intermediates were free to escape, the cells would have to synthesize intermediates for some time to build up a suitable concentration in the entire medium, before protein and nucleic acid synthesis could proceed.

Though this crude model is all that is necessary in considering the chemical changes which occur during metabolism, it is not adequate if free amino acids are assumed to be metabolic intermediates. Direct measurements have shown that the cell wall presents no significant barrier to many molecules, including several amino acids. The whole usefulness of the competition method depends on the ability of exogenous competitors to reach the regions which cause alteration or incorporation into macromolecules. Furthermore, the study of extracellular products (Year Book No. 52) shows that amino acids such as glycine and glutamic acid stay within the cell or diffuse out into the medium depending on the concentration in the medium. Clearly, the cell is in intimate contact with its environment, and most if not all of the small molecules pass freely through the cell membrane.

We have seen no evidence, however, that any concentration of amino acids must be built up in the medium before protein synthesis can occur. When a growing culture is washed and inoculated into a fresh medium, its growth rate shows no alteration. Many cases have been found where the cells utilize an exogenous amino acid (even when this is present only in trace quantities) in quite different ways from endogenous material. In these cases it is certain that the material formed in

the cell does not diffuse out into the medium and back before being used for protein synthesis.

Accordingly, we have here a paradox: amino acids are free to diffuse into and out of the cell, but in many cases they do not diffuse out. In a similar but perhaps less striking way, many compounds which seem to be intermediates (fructose-6-phosphate is a good example) do not compete as might be expected. Other compounds which do diffuse inward and do compete still do not compete to the degree which would be expected on the basis of the simple model.

To resolve this paradox and to give a better interpretation of observations, the model of cellular organization must be revised. One possible revision is to assume that, even though the cellular membrane is permeable, certain regions of the cell are surrounded by impermeable or selectively permeable membranes. At first sight this hypothesis is attractive because it permits arbitrary limitations on the access of exogenous compounds to the reactive centers of the cell and provides a mechanism for holding endogenous metabolites within desired channels. When examined more critically, however, this hypothesis becomes untenable.

For example, if it is used to explain the behavior of threonine, it must be assumed that the region where threonine is synthesized, together with the regions where threonine is used for protein synthesis, is enclosed by a membrane of very peculiar properties. Exogenous threonine must be able to penetrate this membrane, as exogenous threonine can be utilized for protein synthesis. Threonine formed within the membrane must not be allowed to leak out, or it would encounter the enzymes which convert it to glycine. The region enclosed must include the entire group of reaction sites at which proteins are formed, because the threonine is needed at all of them. A similar region would be required for lysine, as the same distinction exists between endogenous and exogenous lysine.

These two regions must be overlapping, since both threonine and lysine are required for protein synthesis. As such a situation is clearly absurd, the concept of

internal permeability barriers must be discarded as a mechanism for keeping endogenous amino acids within the cell.

These experiments had extended from 1948 to 1954 with complete consistency. The report in Year Book 54 was therefore the outcome of a period of turmoil.

PERMEABILITY

Escherichia coli has a cell wall which can be separated from the rest of the cell material as a structural entity. Consequently, when a compound added to the growth medium is found to be biologically inert, one possible explanation is that it cannot penetrate the cell wall. Also, when a number of compounds are found to be concentrated within the cell, it must be decided whether they simply cannot penetrate the membrane to diffuse out, or whether some kind of binding is involved.

During the past years the permeability of *E. coli* and *T. utilis* has been measured with many low-molecular-weight compounds and ions. In practically all cases the cell wall appears to be permeable and to have little effect on the passage of material in and out of the cell. These permeability measurements, however, are never finished. Whenever new compounds are investigated, their ability to permeate cell walls needs to be determined. Accordingly, some additional permeability measurements have been made during the year which supplement those of the past. Some further investigation has been carried out with *Staphylococcus aureus*, which is entirely different from *E. coli* in regard to permeability. During these experiments it was noticed that the permeability of *E. coli* to SO_4^- ions is not the same as previously measured. In contrast, the measured permeability to many other small molecules and ions has not altered.

Very briefly, the method used for measurements of permeability is as follows: A

weighed, centrifuged pellet of cells (0.5 to 1.0 gm) is suspended in a known volume of solution (0.5 to 1.0 ml) containing a labeled compound; the suspension is centrifuged, resuspended in unlabeled solution, and again centrifuged; assays for the concentration of the labeled material are carried out on each of the solutions, and in this way the fraction of the volume of the pellet which is accessible to the labeled compound is measured.

For *E. coli* the space accessible to practically all low-molecular-weight substances is 75 per cent of the cell volume, which is roughly the same as the water content of the cell. Thus, the term "water space" has come into use. Since different values have been obtained for different substances in *S. aureus*, the term "accessible space" will be used to avoid confusion, and the volume of this space will be expressed in percentage of the volume of the centrifuged cell pellet.

Staphylococcus aureus. Accessible-space measurements have been carried out on *Staphylococcus* with the following substances (the results being shown in parentheses): gamma globulin (26 per cent); dextran, a high-molecular-weight polysaccharide (29 per cent); SO_4^- (39 per cent); glutamic acid (38 per cent); D_2O (80 per cent). These values represent the averages of many determinations, with some variations in growth conditions and in concentration of the substances concerned. There seems to be very little doubt that the fraction of the pellet volume accessible to SO_4^- and glutamate is

larger than that accessible to the high-molecular-weight substances. These results indicate that a pellet of *Staphylococcus* cells contains a true intercellular space (accessible to all substances) of 26 to 29 per cent of its volume. In addition to this, there is a small volume (about 10 to 15 per cent) of unknown nature (possibly a slime layer) which is accessible to $\text{SO}_4^{=}$ and glutamate. Another 40 per cent of the pellet is accessible to D_2O but not to the other substances tested. Finally, there is a small volume (20 per cent) which is completely inaccessible and roughly equivalent to the volume of the cellular material when dried.

If a large part of the cell volume were enclosed, as these data indicate, in a semi-permeable bag, it would be expected that shrinkage would occur in high salt concentrations. Ten per cent NaCl causes a shrinkage to about half the original volume of the pellet, and the original volume is approached after washing in isotonic solution.

Torulopsis utilis. Measurements have previously been made of the accessible space of *T. utilis* to fructose (72 per cent), valine (68 per cent), and aspartic acid (65 per cent). These measurements show that this yeastlike cell is freely permeable to small molecules and thus is more similar, as regards permeability, to *E. coli* than to the gram-positive *Staphylococcus*.

Escherichia coli. The accessible space of *E. coli* has been directly measured for valine (70 per cent), proline (70 per cent), sucrose (74 per cent), fructose (76 per cent), and glucose (72 per cent). In addition, the kinetics of amino acid uptake give evidence supporting the concept that the cell is permeable—i.e., that low-molec-

ular-weight compounds enter by free diffusion.

Experiments on the kinetics of proline uptake, similar to those described in a previous section, but at very low proline concentrations, indicate that the rate of uptake of proline is within an order of magnitude or so of the rate of free diffusion of proline in water solution into a sphere of water one micron in diameter. The question whether any other process sets a limit on the rate of uptake of proline cannot yet be resolved.

Over a period of several years' work on the permeability of the *E. coli* cell in this laboratory, literally many dozens of individual measurements of the space accessible to the sulfate ion have been made using these same procedures, always with the same result, 75 ± 5 per cent. Since last fall, however, the measured values have centered around 32 per cent with a spread of ± 5 per cent. Numerous attempts have been made to track down the cause of this change, without success. The same value was found for cells grown from an inoculum of strain B supplied by the American Type Culture Collection and for cells grown from a freeze-dried preparation of our usual strain B stored since 1949. The change is apparently not caused by trace substances now present in our growth media, and, as far as we know, has occurred only for the sulfate ion.

The fact that the permeability characteristics of *E. coli* are very different from those of the *Staphylococcus* is shown by the observation that there is no measurable shrinkage of *E. coli* in high salt concentrations, whereas with *Staphylococcus* there is a partly reversible shrinkage of about 50 per cent.

The finding that *E. coli* was not freely permeable to $\text{SO}_4^{=}$ raised doubts about its permeability to other compounds. Further investigation using new techniques led to a considerably altered concept of permeability reported in Year Book 55 (June 1956).

Permeability. A renewed interest on the part of biochemists in the permeability of the bacterial cell is becoming increasingly evident. In our own work, measurements of the permeability of *E. coli* have taken on a new importance since they contribute to the understanding of the mechanisms for the formation and maintenance of metabolic pools.

An advance in the technique of permeability measurements, through the use of filters, has made possible the measurement of the amount of a compound that leaves the cell within a few seconds, and has disclosed errors in the previous interpretations of accessible space measurements.

The direct measurement of cell permeability is carried out essentially as follows: A weighed pellet of cells (with no energy source and no growth occurring) is suspended in a small volume of buffer containing a labeled compound, and centrifuged. The pellet is resuspended in unlabeled buffer and again centrifuged. Assays for the concentration of the labeled compound, carried out on the original radioactive solution and each of the supernatant solutions, make it possible to determine the quantity of labeled compound entering or leaving the cell during the 5 to 10 minutes required for suspending and centrifuging. From these results the fraction of the cell volume which is accessible to the compound may be calculated, the concentration within the accessible volume being assumed to be the same as that outside the cell. The meaning of such a volume calculation is discussed below.

The question arises, however, whether some of the compound is held within the cell in a loosely bound form similar to the TCA-soluble pool described earlier in this report. In order to estimate "bound" material a sample of the labeled suspension (just before centrifuging) is diluted into a large volume of unlabeled buffer and instantly filtered on a collodion membrane filter. The time from dilution to completion of the filtering is 5 to 15 seconds. In this time, diffusion equilibrium will be established for molecules free to diffuse from the cells, since the calculated half-

time for diffusion equilibrium out of a 1-micron-radius sphere of water is less than a millisecond.

When this procedure is carried out with high concentrations of an amino acid, such as proline or valine, it is found that two-thirds of the amino acid that entered the cell in the first suspension has left the cell before filtering can be completed, that is, within 15 seconds. When filtering is performed at later times after dilution, it is found that the remaining one-third of the amino acid leaves the cell slowly with a half-time of about 15 minutes. Points taken at close intervals within the first minute show no significant change occurring.

Thus, a clear separation can be made between a fast component and a slow component. The fast component is assumed to be amino acid that is free to diffuse from the cell, and the slow component is presumably bound in a way similar to the metabolic pool material. We are therefore led to define the fraction of the cell volume that would contain the freely diffusible quantity, at the external concentration, as the volume accessible by free diffusion for the particular compound. The values for the accessible volume based on this new definition differ in many cases from previously published values which were not corrected for the "bound," slowly moving material. Earlier measurements, uncorrected for the "bound" amino acids, indicated accessible spaces of about 70 per cent of the cell volume for amino acids. With the new definition the accessible space for free diffusion of amino acids appears to be about 40 per cent of the cell volume.

A schematic diagram of the cell, showing the volumes accessible by free diffusion, can now be constructed as shown in figure 32. It will be noticed that there is a volume inaccessible even to D_2O which corresponds roughly to the volume of the dried cell material. In addition, there is a volume which is inaccessible by free diffusion to amino acids. The volume accessible by free diffusion for sodium appears to be somewhat larger than that for amino acids or $PO_4^{=}$ (about 55 per cent). It

should be pointed out that the volume accessible to D_2O has not been measured with the new rapid technique, and it is therefore conceivable, though unlikely, that part of the 75 per cent volume shown as accessible to D_2O is not in fact accessible by free diffusion.

The meaning of the concepts *permeable*, *accessible*, and *volume*, as they have been used above, must now be considered in the light of the new evidence presented. Growing cells hold in the pool 1000 times more amino acid than would be contained in the accessible volume at the external concentration. In fact, cells can take up valine into the pool at a rate approaching the maximum set by diffusion toward the cell. Thus, in referring to the permeability of a cell, it must be made clear whether an active or a passive process is being considered. To avoid connotations resulting from previous use or misuse of the word permeability, we prefer the word accessible as in the phrase "accessible by free diffusion."

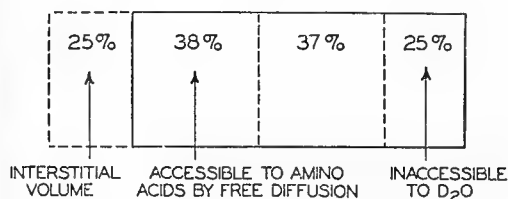


FIG. 32. Schematic diagram of the calculated accessible volumes of the cell.

Careless interpretation of accessible volume measurements, in attempts to judge the availability of a compound for cell metabolism, must be avoided. For instance, the accessible volume by free diffusion for SO_4^{2-} is only 15 per cent of the cell volume when cell metabolism is at a low ebb in thick suspensions without energy source. Growing cells, however, take up all the sulfur needed for full growth rate even in very low sulfate concentrations. And, strikingly, sulfur-starved cells will take up SO_4^{2-} at a rate approaching the maximum rate permitted by free diffusion toward the cell. It may *not* be concluded, therefore, that a compound showing a low

accessible volume is not readily available for metabolism. On the other hand, a compound showing a large accessible volume may be unavailable to the cells' metabolism. The lack of correlation between permeability or accessible volume and the rate of entry of a compound into metabolic processes is not generally realized.

The volume of the cell calculated to be accessible to the given compound by free diffusion does not necessarily correspond to a real physical volume within the cell. The calculation depends on the assumption that the concentration within the volume is the same as that outside the cell, and is thus useful as a simple description of the experimental results. The fact that the accessible volume is independent of concentration supplies partial justification for the assumption.

There are, however, at least four ways in which an accessible volume, 38 per cent of the cell volume, might be interpreted: (1) the concentration over the whole cell is 38 per cent of that outside—in effect a concentration-independent partition coefficient is postulated; (2) the concentration over the whole cell is zero, and the compound is adsorbed on the surface of the cell; (3) the concentration within part of the cell is equal to that outside, but the remainder of the cell is walled off, for example, by a plasma membrane; (4) the concentration in regions scattered throughout the cell is equal to that outside, but a large number of small regions are inaccessible by free diffusion to the compound. These regions may be the individual macromolecules, or larger structures such as the "microsomes" which have been extracted from *E. coli* and photographed by Schachman. It is worth noting that the aggregate volume of these 15-millimicron ribonucleoprotein particles is about 35 per cent of the cell volume.

Finally, these different descriptions may be mixed according to taste. One must, however, choose a description that fits what we know of the real physical organization of the cell and serves as a satisfactory framework for the interpretation

of other evidence. The general evidence with regard to metabolic pool formation and the osmotic characteristics of the pool,

together with what is known of the physical organization of the cell, leads us to favor the fourth description.

A similar statement was inserted at the end of the chapter on permeability in the second printing of Studies of Biosynthesis in E. coli.⁴ To this day we do not understand how it was possible to obtain consistent results indicating one conclusion and thereafter consistent results indicating a different one. This experience has had a marked influence upon us here, both in calming down enthusiasm over initial results and in creating skepticism in regard to reports in the literature.

The early results and concepts of a freely permeable cell have continued to influence our thinking. When cells concentrate small molecules we tend to wonder what binding forces hold the small molecules rather than what transport mechanism brings them into the cell. If necessary, carrier molecules are invoked into models of pools, but they are regarded as intermediaries between the outside free molecules and the loosely bound state within the cell. This different view may or may not have validity, but at least it is different.

It is also of interest that the first mention of "microsomes" (now called ribosomes) appears as speculation whether these particles could account for the inaccessible regions of the cell.

E. Synthesis of Small Molecules in the Intact Cell

The Biophysics Section came into a true focus for the first time upon the synthesis of amino acids and nucleotides. Year Book 50 describes two essential ingredients for these studies, which were to be predominant for the next four years.

One of them was isotopic competition. This relatively simple concept was first needed to explain the reduction in the incorporation of $P^{32}O_4^{=}$ that occurred when glucose-6- P^{31} was added to a medium containing $P^{32}O_4^{=}$.⁵ It was more clearly recognized and further elaborated in studies of $S^{35}O_4^{=}$ incorporation.^{6, 7} In the following years it was extensively used to avoid the necessity of kinetic measurements for the determination of precursor product relationships in biosynthesis of small molecules.

The other was the technique of paper chromatography, which had recently been developed by Martin and Synge. This elegant method supplemented by radioautography allowed a rapid analysis of small quantities of hydrolyzed cells and quantitative measurement of the radioactivity of various fractions.

The emphasis on amino acid synthesis resulted from a conjunction of the work of Cowie and Bolton on the incorporation of $SO_4^{=}$ into cystine and methio-

nine with the exploratory experiments of Abelson, who wished to see which compounds were formed from $C^{14}O_2$.

The following years (to 1955) brought studies of a variety of labeled nutrients (acetate, glucose, amino acids, nucleosides) in competition with unlabeled compounds.

This work was summarized in Studies of Biosynthesis in E. coli.⁴ It added little to the qualitative knowledge of biosynthetic pathways already accumulated at the time. Its main contribution was to integrate a variety of individual reactions into a picture of the whole process and to introduce a quantitative aspect. For example, the reactions of the Krebs cycle were known, but these studies showed that the Krebs cycle, as it operated in growing bacteria, though contributing relatively little to the energy of the cell was essential in the formation of synthetic products. The rates of various reactions were indicated in flow diagrams outlining the main flows of material (Studies of Biosynthesis in E. coli, p. 425).

In addition, the concepts of cellular regulation were recognized. Changes in the enzymic constitution of a cell in accord with the ingredients of the medium were well known at that time, but more rapid changes were needed to interpret the results of isotopic competition. These were recognized as inhibition of synthesis (p. 412) and were designated a "chemical response." The "chemical response" (now known as enzyme inhibition or feedback control) was set in contrast to changes in enzymatic constitution designated the "biological response" of the cell (p. 435). The quantitative studies of the metabolism also made clear the flexibility of the metabolic system (p. 435) and the couplings that exist between various systems (p. 436).

These studies of the biosynthesis of small molecules showed the value of concentration on a single problem. One finding reinforced another and brought forth the first valuable contribution of the Biophysics Section to the current of biological thought. It also provided a firm basis for the continuation of these studies in other organisms or for extension into other areas.

F. Pools

With the conclusion of work on the E. coli monograph in the fall of 1954 the Biophysics Section was free to renew experimental work. The methods and techniques developed for the study of the biosynthesis of small molecules could be applied to other organisms or to varied metabolic conditions. Alternatively, the work could proceed in new directions.

The first intimation of the shift in objectives appeared in Year Book 53, p. 77 (June 1954):

The past year has marked a period of transition for the biophysics group. We have attempted to shift the direction of our work from the study of the biosynthesis of amino acids and nucleic acid bases toward a study of synthesis of the macromolecules. Several exploratory ventures were carried out in this field, including studies of the role of peptides in protein synthesis, the kinetics of protein and nucleic acid synthesis, and the incorporation of tracers by cell fragments. In addition, considerable progress was made in

learning how the components of nucleic acid are assembled.

It has been difficult, however, to break away completely from the previous studies of amino acid synthesis. A large share of the available time was spent in the preparation of a monograph describing previous work with the smaller molecules. Many experiments had to be carried out to provide additional data required for the monograph. Some of these experiments showed unexpected features which required further investigation.

Not until June 1955, however, was the transition complete.

During the past year the biophysics section, a small group of physicists who have undertaken the study of order and process in living materials, has made a major shift in its objectives. Previously we were content to study ways in which cells synthesize small molecules for subsequent incorporation into macromolecules. This year we have attempted to learn something of the processes by which the small-molecule building blocks are assembled to make the biologically active molecules of protein and nucleic acid. We were persuaded to make this shift not so much because the study of small molecules was complete, as because the new field offered a greater challenge together with some hope that significant progress was possible.

During the past few years several promising models for the synthesis of macromolecules have been put forward. The Watson-Crick structure for deoxyribose nucleic acid (DNA) provides a model of a molecule which is inherently capable of being precisely duplicated. It consists of two spiral chains, each of which carries the same information. Consequently, if it is split into two halves, each half has the capacity for accumulating the missing material and being reconstituted into the original form.

This model is particularly useful in the attempt to understand how certain virus particles are reproduced and how genes are duplicated and transmitted down through the generations with few changes. It is

less useful, however, in giving any clear picture of synthesis of the other biologically important macromolecules, ribose nucleic acid (RNA) and protein. Somehow the DNA molecule must have an influence in determining what types of RNA and protein molecules are synthesized, or it would have no genetic significance. Furthermore, RNA must have some of the same properties, since RNA seems to be the active ingredient of many plant viruses. Finally, specific proteins must be synthesized; possibly RNA acts as a template for arranging the order of amino acids in a peptide chain.

Certain specific questions can be asked which have a bearing on these general ideas. Are amino acids utilized as such, or are they first linked into small peptides? If a template is involved in protein synthesis, can the adsorption of amino acids on the template be observed? If so, can the nature of the template molecule and the type of binding be determined? Experiments which answered these questions would clarify many of the fuzzy areas of macromolecule synthesis.

During the year we have studied the kinetics of protein formation in bacteria and yeast. Specific adsorption of amino acids as a first step in their incorporation into proteins has been observed. In the coming years, further studies of the adsorbed amino acids may reveal the nature of the templates used by the cell for protein synthesis.

During the fall of 1954 Cowie and Floyd Rector began to examine the metabolic pools of yeast. TCA extracts of these organisms were chromatographed, and streaks resembling a mixture of peptides appeared on the paper. Peptides were of considerable interest at that time because the theory that proteins were assembled from peptide precursors still had considerable support. The kinetics of the material in the streaks were studied to determine whether they represented intermediates of protein synthesis. The streaks eventually turned out to be artifacts caused by traces of TCA remaining in the extracts. When the TCA was removed the streaks were replaced by a variety of amino acid spots which were protein precursors and showed a number of other unexpected characteristics.

A similar interest in the metabolic pools of E. coli had its origin at a conference at Valhalla, New York, sponsored by the Yale Biophysics Department in January 1955. Drs. E. Pollard and H. Morowitz attempted to calculate whether the rate of protein synthesis was limited by the rate of diffusion of amino acids to the synthetic sites. Unfortunately the concentration of amino acids in the cell was not known.

Taylor⁸ had reported negligible quantities of amino acids in washed Gram-negative bacteria in contrast to the large accumulations found in Gram-positive cells. It therefore appeared that the concentrations were extremely low, since the effectiveness of water washing in removing pools was not known.

Britten and Roberts returned from the conference and started a new series of measurements of the nonprotein amino acids of E. coli. Considerable quantities and rapid uptakes were observed in the first experiments that used centrifugation to separate the cells from the media. The rapid kinetics demanded quicker methods of sampling, and so the filter technique was devised by Britten. This simple scheme allowed samples to be taken in a matter of seconds and has continued to be of the greatest usefulness (II.B.1).

By April 5, 1955, some of the basic features of pool formation in E. coli had been discovered, and they were reported at the Symposium on Structure of Enzymes and Proteins sponsored by the Oak Ridge National Laboratory.⁹ Cowie, at the same time, described concurrent work on yeast pools.¹⁰ A more complete report of the E. coli work was presented at the April 25, 1955, meeting of the National Academy of Sciences and published in the Proceedings of the National Academy of Sciences (II.B.1). Very similar experiments were being carried out in Paris by G. N. Cohen and H. B. Rickenberg, who gave a preliminary report of their results on May 23, 1955.^{11, 12}

At the time of the first work on amino acid pools the concept of free permeability of the cell to amino acids still flourished. Although some doubts had been introduced by the experience with $\text{SO}_4^{=}$, the space accessible to amino acids remained at 70 per cent. This figure was not revised downward until the following year. The results were therefore interpreted in terms of free permeation followed by adsorption on sites. For small pools, at least, the sites were specific and might well be the actual sites for protein synthesis.

The Pasteur group, in contrast, interpreted their results in terms of a simple model comprising an impermeable cell wall, a pump ("permease"), and a leak.

As often happens, further experiments revealed additional complications, and both the simple early models failed. A "carrier" had to be added to the adsorption model, and other modifications had to be introduced in the "permease" model. To quote from Year Book 56, p. 119:

AMINO ACID POOLS IN *Escherichia coli*

Further studies have been carried out on the amino acid pools of *E. coli* in order to understand the mechanisms of pool formation and maintenance. The work has been guided by the general idea that a detailed understanding of the first step in amino acid incorporation by the cell will supply a foundation for investigations of the later steps leading to macromolecule synthesis, and may supply clues to the mechanisms involved in the later steps.

Pool formation is an expression of the ability of the cell to obtain nutrients present at very low concentrations in the environment and to supply them to the synthetic machinery at high concentrations. This, perhaps, allows significant simplification of subsequent problems of macromolecular synthesis.

The principal question is whether the internally concentrated substances are free in solution within the cell or held in a more complex fashion. If the pool is simply a concentrated solution that pervades the cell, then the synthetically active structures

within the cell are bathed in this solution, which is thus the "medium" in which synthesis occurs. On the other hand, the amino acids of the pool may be more closely associated with the substructures of the cell responsible for protein synthesis. They might be trapped in such substructures (as in a brush heap) or be bound to them by labile chemical bonds. In the latter case it would be highly important to know the nature of the binding sites and how intimately they are related to the synthetic activities.

The experimental work described below was aimed at distinguishing between these alternatives. Although no directly conclusive experiments were devised, the totality of the experimental evidence obtained showed that various simplified models based on these alternatives were inadequate. The elaboration of the properties of the pool obtained by these studies provides a list of critical requirements that must be met in the formulation of any satisfactory model.

At present the equations of the two models are quite similar, as they must be if they are to describe the same phenomena (see section II.B.2). We still find the word "permease" repugnant, as it implies the existence of a protein enzyme that had not been (and still has not been) demonstrated. Some of the properties of the carrier seem difficult to attribute to a protein molecule fixed in the cell membrane.

As studies of the pool continued through 1955 and 1956, it became increasingly apparent that the pool was not held by the protein-synthesizing sites. By 1957 attention shifted to ribosomes and their synthesis.

An exact knowledge of the properties of the pool is, however, necessary both for the design of experiments and for the interpretation of results in the study of the kinetics of RNA synthesis. Further studies of the nucleotide pool were therefore carried out in 1961 and 1962. It was gratifying to find that the equations of the carrier model, which had been developed to account for the behavior of amino acids, were adequate to describe the entry of nucleotide bases. The work on pools is described in section II of this volume.

In retrospect, the years spent in studying pools were a necessary prelude to the study of macromolecular synthesis. In general the pool stands between the medium and the sites of synthesis. The properties of the pool must be understood to devise ways of avoiding the kinetic delays the pool can introduce.

G. Ribosomes

Interest in ribosomes developed in part from their role in protein synthesis, which had been demonstrated in mammals, but more from the possibility that they might play a part in holding amino acid pools. In 1956 difficulties were apparent in both the adsorption and the pump models of pool formation, but the carrier model had not yet been formulated. This view is stated in Year Book 55, pp. 134-136:

The experimental facts outlined above are not sufficient to be assembled into any definite picture of protein or nucleic acid synthesis. Yet it is worth the effort to assemble them into some sort of hazy scheme, partly for the record, to show how near or how far from the final answer we were in 1956, and partly to provide a target for the next year's experiments.

It seems quite certain that exogenous materials are concentrated by the cells before their use for synthesis. Some of the materials are chemically altered and stored in the altered form—thus adenine is taken up by the cells and rapidly converted to ATP. Glucose is rapidly converted to a wide variety of compounds found in the TCA-soluble pool before its carbon appears in the macromolecules. Other materials, amino acids especially, seem to remain unchanged while they are in the soluble fraction. Carbon supplied to the cell as proline is in part converted to glutamic acid and arginine, but most remains as proline and is incorporated into the protein as proline. No activated forms of amino acids have been detected.

Even though some alterations do occur, there is no evidence of any partly formed fragments of protein or nucleic acid. Instead, quite low limits can be placed on the possible quantities of peptides or small polynucleotides, as any sizable pool of these materials could be detected either by chromatography or by a kinetic delay in the incorporation of tracer materials.

Nor is there any unusual distribution of newly incorporated amino acids in what is presumably newly formed protein. Rather, amino acids seem to be distributed at random throughout the peptide chains. Accordingly, we are forced to the conclusion that synthesis of the proteins takes place with great rapidity, and that, in a matter of a few seconds at most, the small building blocks find their proper order and are linked together. This course of events is in accord with the commonly accepted idea that order arises from order, and that pre-existing molecules act as the templates for newly forming ones.

The rapidity of the process is perhaps surprising. There are roughly six times

as many amino acids in the proteins of *E. coli* as there are nucleotides in the RNA, and during a generation the quantity of each doubles. If we assume that RNA acts as the template for protein synthesis, and that two nucleotides are required to determine the proper amino acid for each location, then it would be only necessary for each template to act twelve times during one generation. At 18° C, where the generation time is 3½ hours, 16 minutes could be allowed for the amino acids to find their proper place on the template. Instead, the actual time taken was less than 3 seconds. It is possible that only a small part of the nucleic acid is active, or that protein synthesis takes place during only a small fraction of the generation time. It is also possible that the amino acids move on and off the template by exchange until the instant that the peptide chain is formed. It seems much more likely, however, that whenever a template is exposed it is rapidly covered with amino acids, which then link to form a completely new polypeptide. The time-consuming process could then be the stripping of the protein from the template.

The newly formed nucleic acids have not yet been examined, but the transition from small TCA-soluble nucleotides to TCA-insoluble nucleic acid seems to be equally rapid.

This type of mechanism implies that there is little chance of finding any intermediates and little hope of gaining further clues about the mechanism from an examination of the end products. The behavior of the small molecules before their assembly into macromolecules seems the most likely source of information on the synthetic process. Accordingly, we must consider the properties of the pool materials.

Exogenous amino acids present in the medium are concentrated by growing cells so that the concentration per milliliter of cells rises to several hundred times the concentration in the medium. These concentrations can be explained in terms of adsorption or in terms of a pump mechanism

that transfers the amino acids to an impermeable region of the cells. Several difficulties promptly appear in both models. Adsorption can hardly be the sole mechanism involved, for the following reasons:

1. The quantity of material held within the cells is very large. Several hundred micromoles per gram dry cell of an amino acid can be held, a quantity which is roughly one-third of the number of RNA nucleotides or one-twentieth the number of protein-bound amino acids in the cell. The adsorption of such large quantities seems unreasonable.

2. The interference between similar amino acids does not have the characteristics expected from competition for adsorption sites.

3. Adsorption processes would not be expected to be sensitive to the osmotic strength of the medium.

The pump model also has serious difficulties:

1. An appreciable fraction of the cell is accessible to amino acids by free diffusion; consequently, the pump cannot be considered as simply working to maintain a concentration gradient across the cell wall.

2. An energy source is required to build up the concentration within the cells, but not to maintain an existing concentration. Exchange continues, however, in the absence of an energy source. Therefore, the barrier that maintains the concentration is by no means impermeable, and the pump would have to continue to operate without an energy source to maintain the concentration gradient. Without the energy source it could not build up even a much lower concentration.

3. The release of amino acids by osmotic shock does not have the characteristics that would be expected if the process involved bursting a membrane.

4. It is difficult to see how the maximum capacity of a region within a membrane could increase when the osmotic strength of the medium rises.

In the face of these difficulties with both the adsorption and the pump models it

seems advisable to attempt the construction of a new model that will meet more of the requirements. In mammalian tissue it is the microsome fraction that is the first to incorporate amino acids. Furthermore, the active elements of the microsome fraction are particles consisting of approximately one-half protein and one-half nucleic acid. Similar particles are found in *E. coli*. If all the RNA of the cells is located in particles that are 50 per cent RNA, then about 30 per cent of the cell mass would be found in the particles. Since about this fraction of the cell is accessible by free diffusion to water, but not to amino acids, and since the amino acids might be expected to localize in regions where protein synthesis occurs, it seems reasonable to postulate that the amino acids of the pools may be trapped within the three-dimensional framework of particles.

Not enough is known of the physical chemistry of these organized networks of

nucleic acid and protein to predict definitely what their properties might be, particularly while they are still within the cytoplasm of the cells. It does not seem too unreasonable, however, to suppose that they might exhibit the observed sensitivity to the osmotic strength of the medium. Such structures could provide the needed capacity to hold amino acids, and a degree of specificity might result from the configuration of their interior spaces.

The idea of such a structure as the basic unit for growth is, of course, not new. The feature that has been added is that these particles may also concentrate the precursors of protein and nucleic acid. If this hypothesis turns out to be true, it may be possible to study some of the characteristics of the particles as they exist within the cells by observing which compounds can or cannot prevent osmotic shock. At this stage, however, the main virtue of the model is that it stimulates new experiments.

Early in 1957 arrangements were made to use an ultracentrifuge at the National Institutes of Health. These runs indicated that we would have a continuing need for a centrifuge, and a Spinco model L was purchased. The first pellets were examined both for their content of pool amino acids and for their content of newly formed protein. Contrary to our expectations neither was prominent in the pellets.

The cell juices obtained by breaking the bacteria in the French pressure cell were quite unstable in the usual buffers; aggregations occurred frequently. An assortment of suspending fluids was tested to find a suitable one. Among them was a tris-succinate buffer containing magnesium and manganese, which Spiegelman had used in a cell-free system. Surprisingly, the presence of manganese caused aggregation of the cell juice into round spheres similar to yeast. The fascinating lifelike appearance of these objects which formed spontaneously from cell juices brought a short diversion from the study of ribosomes while their properties were investigated (IV.C.6).

Having pellets of ribosomes available from the model L centrifuge, we then needed to examine them with the analytical centrifuge. Bolton spent the summer at the Rocky Mountain Laboratory of the U. S. Public Health Service, where a Spinco model E was available. With Hoyer and Ritter he soon discovered the importance of magnesium in stabilizing the large ribosomes, which promptly dissociated to 30S and 50S particles when the magnesium concentration was reduced (III.C.1). Similar results using yeast ribosomes were obtained by Chao, whose report appeared in the early fall.¹³

These experiments also showed the variability of ribosomes and the need to monitor preparations with the ultracentrifuge. A model E analytical Spinco was procured for our own laboratory in 1958.

Studies of tracer incorporation were continued. Both chromatography on DEAE columns and sedimentation analysis were employed. By the end of 1957 two macromolecular precursors of the ribosomes had been detected. These results were reported at the Symposium on Microsomal Particles and Protein Synthesis held by the Biophysical Society in Cambridge, Massachusetts, February 5, 1958 (III.C.2).

Nascent protein associated with ribosomes proved harder to detect. After moderately short (4-minute) exposures of growing cells to radioactive amino acids or $S^{35}O_4^{=}$, both the nucleoprotein peak separated by the DEAE columns and ribosome pellets showed specific radioactivities lower than those of the soluble proteins. These difficulties were described in Year Book 57, pp. 138-139:

"In order to examine the precursor-product relationships among the macromolecules of the cell and to assess the possible role of the ribosomes in the synthetic processes of the cell, kinetic studies have been made of radioactive-tracer incorporation into cell fractions separated by means of ion exchange and the ultracentrifuge.

"The kinetic studies of protein synthesis using S^{35} as a tracer are still in a preliminary stage. Exponentially growing cells were exposed to the tracer for varying periods of time and then broken and their constituents separated by centrifugation and chromatography. The specific radioactivity of the protein fractions was measured by TCA-precipitable S^{35} and Folin reaction color. When the cells are exposed to the tracer for a prolonged period (steady state) the specific radioactivity varies throughout the chromatographic elution pattern by a factor of roughly 3, being lowest in the nucleoprotein fraction. These variations are simply due to differences in the sulfur content of the different proteins. Alternatively, cells were grown for three generations in a nonradioactive medium after exposure to the tracer. In this treatment any intermediates that have a rapid turnover should lose their radioactivity. The resulting 'persistent pattern' was entirely similar to the 'steady-state pattern,' and no protein components could be identified as intermediates.

"Growing cells were also exposed to the tracer for short periods. After a 4-minute exposure the resulting 'pulse pattern' was similar to the 'steady-state pattern' except that the radioactivity of the nucleoprotein peak was only half that expected from the 'steady-state pattern.' A similar result was obtained with cells exposed for 4 minutes to a mixture of C^{14} -labeled amino acids.

"It appears from these observations that the nucleoproteins (i.e. CNP) of the ribosomes are not precursors of other proteins of the cell. Possibly, however, newly formed proteins might still be found in association with the ribosomes after breaking of the cells. To test this possibility, a 100,000g, 2-hour pellet containing ribosomes (and contaminating protein) from pulse S^{35} -

labeled cells was analyzed on the column. It was found that the traces of protein eluting at the same salt concentrations as the bulk of the cellular protein had in fact the same specific radioactivity as the total protein of the cell--not the high specific activity indicative of precursors. This failure to observe precursor proteins in association with the ribosomes is not conclusive evidence that such an association does not exist. On the one hand the turnover rate could be so high that very much briefer pulses would be required for the observation, or on the other hand the association may be so labile as to be destroyed by breakage of the cells."

At this juncture K. McQuillen joined the section for a year's sabbatical from the University of Cambridge. A much improved technique of sedimentation analysis coupled with much shorter exposures to the tracer proved adequate to demonstrate the nascent protein (IV.A.1).

The unraveling of the steps involved in ribosome synthesis proved much more difficult. The DEAE column showed that there were two sequential precursors to the ribosomes, but these fractions showed no direct relationship to the fractions found in sedimentation analysis. During 1959 and 1960 a great deal of information was accumulated on the properties of ribosomes, their enzyme content, the composition of the RNA, the relationship of the ribosome content to the metabolic state of the cell, etc. The kinetics remained obscure. No class of particles seemed to be clearly precursors or products. This suggested a circulating system wherein small particles accumulated new RNA to become large particles which then broke down to smaller ones again. Such a system fitted well with the thought that the ribosomes might be formed by autocatalysis. The relationship of DNA to ribosomal RNA had not then been demonstrated.

This confusion is illustrated by a statement taken from a talk given at that time.

"In the experiments mentioned above there is little indication of any role of DNA in the synthesis of proteins or ribosomes. On the contrary, after magnesium starvation, which depletes the content of ribosomes to 5 per cent of normal, the synthesis of ribosomes follows an autocatalytic course.

"There is one slight clue to a role of DNA in the formation of the 20S particles. If these particles were formed solely from the splitting of 30S particles, they would have specific radioactivities lower than the 30S group. In fact, the 20S group invariably has the highest specific radioactivity at early times; thus it seems to be synthesized de novo. Perhaps DNA serves as template for the synthesis of 20S particles that then carry the required information from the DNA to the semiautonomous ribosome system."¹⁴

Late in 1960 these confusing results were cleared up when it was found that a much simpler picture emerged when the sedimentation analysis was performed at low concentrations of magnesium. Precursors and products could be identified and correlated with the fractions isolated by chromatography.

It became apparent that radioactivity appearing in the large particles at early times was due to nascent RNA which adhered to them. In low magnesium the nascent RNA was released and was distinct from the 30 and 50S ribosomes.

The nascent RNA was separated in this fashion and further purified on the DEAE column. Its composition was measured and found to be more like that of ribosomal RNA than that of DNA, in keeping with Bolton's earlier measurement of the total newly formed RNA. These results were reported in February 1961 at a conference in Houston, Texas:¹⁵

"Had the base composition of this particular RNA fraction turned out to show the ratios of DNA, we might have reason to believe that this was, in fact, the 'messenger' RNA which has been postulated. At present, we can only wait to see what further purification may bring. Its composition may indicate a relationship to DNA and an information-carrying capacity."

Further work on the kinetics of incorporation into RNA was completed in time to allow a brief report at the Cold Spring Harbor Symposium in June 1961,¹⁶ and the completed study was described in four papers submitted to the Biophysical Journal in May 1961 (II.C.3; III.B.1; III.B.2; III.B.3).

During early 1961 enthusiasm for the messenger hypothesis of Jacob and Monod was spreading rapidly even though the paper defining messenger RNA had not yet been published.¹⁷ According to this hypothesis the template for protein synthesis should: (1) be a polynucleotide of average molecular weight greater than 5×10^5 but heterogeneous; (2) have a base composition reflecting the composition of DNA; (3) show a loose association with ribosomes; (4) have a very high rate of turnover.

An association of newly formed RNA with old ribosomes in phage-infected cells was demonstrated in a paper in Nature in 1961.¹⁸ The same issue carried an article showing the sedimentation properties of newly formed RNA.¹⁹

This newly formed material was interpreted to be messenger RNA. In contrast, our three years' experience with the same material had convinced us that it was the precursor to ribosomes. These alternative views were presented at the Cold Spring Harbor Symposium in June 1961.^{16, 20}

Another difference developed over the composition of the nascent RNA. Our measurements indicated that the composition differed only slightly from ribosomal RNA, but Gros et al. reported a composition like DNA.²⁰

The composition was finally established by Midgley, who first measured the time course of the apparent composition and then devised a technique for separating two components (III.B.4; III.B.5). These results were verified by the DNA-agar techniques of Bolton and McCarthy (III.B.6). The final outcome was that in growing cells two-thirds of the newly formed RNA is ribosomal precursor and one-third is DNA-like, having the characteristics predicted by Jacob and Monod.

A considerable confusion then developed in the use of the term "messenger." By the original definition it should be applied only to RNA having a DNA-like composition. As late as September 1962, "messenger RNA" was still used as synonymous with "rapidly labeled RNA" even though only a third of the rapidly labeled RNA is DNA-like in composition.²¹

Material having the properties predicted by the messenger hypothesis was then finally demonstrated. Proof of the hypothesis, however, required evidence that this D-RNA did in fact act as the template for protein synthesis. Until mid 1962 the alternative hypothesis that newly formed R-RNA acted as template for most of the protein was equally attractive (III.C.12). This alternative became untenable when it was shown that only a small part of the DNA was related to R-RNA, hence R-RNA could not specify a wide variety of proteins.²²

The messenger hypothesis appears to be established in all its detailed predictions. In fact, the hypothesis was so attractive that it was almost universally accepted as demonstrated a full year before the experimental evidence was accumulated.

The next advance in understanding the mechanism of protein synthesis came with the discovery of polysomes, the complex of several ribosomes attached to a single molecule of messenger RNA.²³ So many clues pointed to the existence of the polysomes that in retrospect it is amazing that polysomes were not observed earlier. On the experimental side there were frequent observations that nascent protein and nascent RNA sedimented more rapidly than ribosomes, particularly when the cells were broken by gentle methods.

McQuillen et al. noted, "There is, however, a slight indication that ribosomes associated with cell membranes may be more active in protein synthesis. When cells were lysed by treatment with lysozyme followed by freezing and thawing about half of the ribosomes were released and most of the remainder were detached from the residual membranous material by passing it through the pressure cell. It was found that the first fraction of ribosomes had only about half the specific radioactivity of the second. Possibly some of the particles exist free in the cell juice whereas others are more or less firmly bound to membranes and are more directly involved in protein synthesis" (IV.A.1).

Spiegelman had found that the rapidly sedimenting fraction was active in the in vitro synthesis of macromolecules.²⁴ Spiegelman and Roberts completed a paper describing experiments showing the large fraction of newly incorporated radioactivity that is found in a rapidly sedimenting fraction. This paper was never submitted for publication because the results were erratic and the procedures for obtaining the fraction were not reliable. In retrospect it seems likely that ribonuclease was breaking up the polysomes of the heavy fraction. At the time the association was thought to be with membranes, and the erratic results were attributed to the lability of complexes with lipides.

As soon as the concept of messenger RNA was introduced, other clues to the necessity of polysomes became available from theoretical considerations.

The RNA template was too large for a one to one association with ribosomes; it was long enough to wrap six times around. Furthermore, the cell contained far fewer template molecules than ribosomes. These difficulties were summarized as follows:

“The second question concerns the apparent excess of ribosomes. If the fraction E_D acts as the sole template material, there are eight 70S ribosomes lacking template material for each ‘active’ 70S complete with template and engaged in protein synthesis. Even if the whole eosome fraction is equally active as template, there is still an excess of 3 to 1. Furthermore, these ratios are calculated for an assumed template of 600 nucleotides. If the templates are in fact larger, the excess of ribosomes is correspondingly greater.

“Since the ribosomes constitute at least 20 per cent of the cellular material, a mutation which dispensed with excess production of ribosomes should bring a 15 per cent increase in growth rate. Considering the elaborate control mechanisms which have evolved to prevent the production of unneeded proteins, it seems likely that a similar mechanism would limit ribosome production if there were a true, and not just an apparent, excess.

“There are several interpretations which can account for the apparent excess. The unit active in protein synthesis may involve more than one 70S ribosome. An excess of ribosomes may be needed to provide a rapid transfer of template material from its site of synthesis on the DNA to ribosomes. If most of the ribosomes were already occupied, the template might remain on its site of synthesis, limiting the activity of the gene. Alternatively, the release of templates without rapid attachment to ribosomes might lead to their degradation or to the formation of secondary structures which would interfere with activity as templates.

“Finally, it may be that the release of nascent protein is a slower process than its formation. If so, the nascent protein could be left on the ribosome while the template moved to another ribosome to form the next polypeptide strand. This view gains some support from the fact that the eosomes are less firmly bound to ribosomes than is nascent protein. Also the quantity of nascent protein exceeds the quantity expected to be associated with templates.”²⁵

The failure to reach a conclusion, which in retrospect should have been obvious, provides a warning. Alternative models should be carefully considered until one model or another is completely satisfying in all respects. The search for better models should be encouraged. Speculation like that of VI.K and L may have some useful result.

H. Conclusion

In addition to the four topics outlined above, other aspects of protein synthesis have been continually under investigation. The coupling between synthesis of protein and nucleic acid was studied first in 1951²⁶ and it remains a subject of great interest. In the early period 1950-1955 a number of experiments were carried out in efforts to verify or rule out the role of small peptides in protein synthesis.

Studies of the incorporation of analogs began with an observation of Cowie and Bolton that selenium could substitute to a limited extent for sulfur. Bolton then switched to a collaboration with Dr. George Mandel in the study of the effects of 6-mercaptapurine. Cowie resumed the work on selenium in collaboration with Dr. George Cohen during a visit to the Pasteur Institute in 1956 and continued the investigation of other analogs with Dr. Cohen when he returned the visit in 1957. Out of this experience developed a number of other experiments with analogs (IV.B).

Studies of enzyme induction have indicated how one particular enzyme is formed for correlation with tracer studies which only show average rates. An attempt was made in 1955 and again in 1960 to isolate the template for a particular enzyme. The fractionation methods available then were inadequate, but the same experiment is again under way.

At present the experimental work is dominated by the DNA-agar column. This device has proved to be extremely versatile in providing answers to many questions previously not amenable to experimental attack. The range of problems extends far beyond the realm of the single cell. It is valuable in measuring the variety of RNA produced; thus it quantitates the number of RNA copies produced by different portions of DNA in single cells and extends into problems of differentiation when RNA's produced by different organs are compared. DNA's of different origin can be compared on the column; thus the relation of virus to host and the relatedness of different organisms (taxonomy and evolution) can be explored. These activities, the subject of another monograph, have initiated a new phase of the work extending into more varied aspects of biology.

Some general conclusions on the activities of the group are perhaps in order, though these views are entirely subjective. Throughout this entire period there has been no formal program or any direction of the work, but some underlying patterns can be perceived. Perhaps the best analogy is to a group of prospectors who explore for gold. When one finds a rich lode the others leave their exploratory work to help dig out the gold already located. As the lode is mined out the separate exploratory ventures are resumed.

The most productive periods are naturally those of concentrated focus on a narrow subject. Each finding helps the whole effort, and new knowledge accumulates exponentially. Equally important, however, are the wide-ranging

exploratory efforts, for without them there would develop no points for concentration.

One vital factor is the composition of the group. In university departments each senior man is surrounded by students, and the interchange tends to be from professor to student. Here we have had more of an exchange between equals, which has led to rapid and strong interactions. In fact, many of the most useful ideas have been the result of such interactions, and frequently it is difficult to trace the origin of an idea.

The size of the group has varied from four to ten active investigators. Four appeared to be too small, too close to the limit of the "critical mass." With as many as ten there develops some difficulty in knowing intimately what everybody else is doing. Six to eight seems an ideal number.

Technical support has been minimal: three girls at the most for the entire group. At periods of high productivity more could have been done, given more help. At other times there was no need to think up experiments simply to keep a technical staff occupied. Furthermore, complete contact with the experimental details is unavoidable when the work is done in large part by the investigator himself.

Most important, again from the subjective view, has been the satisfaction of working with associates who were close friends as well as collaborators.

Needless to say, no group can operate without the full support of its institution. In this respect we have been most fortunate. From the day that Dr. Bush approved the initial experiment of converting a few nuclear physicists into biologists the Carnegie Institution has given us confidence that it would back our efforts and tolerate our failures. The same attitude continued when Dr. Haskins succeeded Dr. Bush as President of the Institution. Our requests for additional equipment or technical support have been granted without delay; there has been no lack of interest in the results. The same climate has prevailed within our department. Dr. Tuve initiated the activities in biophysics and participated fully in the early stages which established the course for later years. Since then he has always found time to follow the developments closely, to provide encouragement, and to give advice when needed. Above all he established the atmosphere of friendly cooperation in research that has been our principal asset throughout the years. Richard B. Roberts.

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APPENDIX 1. BIOPHYSICS PERSONNEL 1946-1964

Staff

P. H. Abelson	D. B. Cowie
E. T. Bolton	B. J. McCarthy
R. J. Britten	R. B. Roberts

Senior Fellows
Research Associates

D. Axelrod	B. H. Hoyer
G. N. Cohen	F. T. McClure
H. H. Darby	K. McQuillen
L. B. Flexner	M. Miranda

CIW Fellows

A. I. Aronson		J. J. Leahy
J. A. Boezi	J. D. Duerksen	H. M. Lenhoff
T. J. Byers	Y. Kato	S. Løvtrup
E. H. Creaser	E. S. Kempner	J. E. Midgley

Visiting Investigators

P.-A. Albertsson	W. T. Ham	R. T. Nieset
B. W. Catlin	R. W. Hendler	E. C. Pollard
A. C. R. Dean	Y. Hotta	F. Rector
H. de Robichon-Szulmajster	R. McAfee	I. Z. Roberts
W. R. Duryee	H. G. Mandel	S. Spiegelman
E. Epstein	J. Marmur	H. J. Vogel
J. B. Flexner	A. T. Ness	C. A. Williams

Assistants

E. Aldous	W. Griffin	P. Roddy
R. E. Bresnahan	D. Johnson	M. K. Sands
J. H. Eames	S. Lohman	A. Shirven
E. F. French	M. Melean	E. Stern
J. M. George	B. D. North	B. P. Walton
J. Gray		J. Young

Summer Students

R. A. Ator	L. T. Comly	A. M. Reynard
J. H. Brazinsky	M. A. Healy	C. Stroebel
M. Buchwald	F. R. Norcross	T. W. Tuve
	J. Randall	

APPENDIX 2

Publications of the Biophysics Section
Department of Terrestrial Magnetism, Carnegie Institution of Washington
(Abstracts and meetings largely omitted)

1931

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